THE EFFECTS OF *CARALLUMA FIMBRIATA* ON APPETITE BEHAVIOUR AND ASSOCIATED NEURAL PATHWAYS IN PRADER-WILLI SYNDROME

By

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In total fulfilment of the requirements of the degree of

Doctor of Philosophy

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To Adam

For all the moments you supported me during this long road,

Thank you! I love you, your vision, your passion,

Your awe inspiring curiosity and awareness.

You help me believe anything is possible xxx.

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I. Doctor of Philosophy Declaration

"I Joanne L. Griggs, declare that the PhD thesis entitled the effects of *Caralluma fimbriata* on Appetite Behaviour and associated Neural Pathways in Prader-Willi syndrome, is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of another degree or diploma. Except where otherwise indicated, this thesis is my own work".

Joanne Griggs [redacted] Date 03/06/2016

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II. Acknowledgments

Thank you, to my daughter Mia for your living example of persistence without complaint. You are teaching us all so much. To my husband Adam you are the other half of this work. I'm so grateful for your efforts, your support and your daily reminders of magic. Ruben your compassion, humour and encouragement are so precious to us all. Your smile, and creative talent always balances our journey towards the vision of full potential for all.

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III. Publications and Presentations

GRIGGS, J. 2010. *Miracle in Potential,* Melbourne, Australia, Braidwood Press. Launched by the Honourable Bill Shorten, Leader of the Opposition Australian Government, the then Minister for Disabilities. Also forwarded by the Director of Developmental Medicine, The Royal Children's Hospital A/Prof. Dinah Reddihough 2010

GRIGGS, J. L., SU, X. Q. & MATHAI, M. L. 2014., Presentation: The effects of *Caralluma fimbriata* on appetite behaviour and associated neural pathways in Prader-Willi syndrome (PWS). International PWS Organizations (IPWSO) Conference, Cambridge University, U.K.

GRIGGS, J. L., SINNAYAH, P. & MATHAI, M. L. 2015a. Prader–Willi syndrome: From genetics to behaviour, with special focus on appetite treatments. *Neuroscience & Biobehavioural Reviews,* 59**,** 155-172.

GRIGGS, J. L., SU, X. Q. & MATHAI, M. L. 2015b. *Caralluma fimbriata* supplementation improves the appetite behaviour of children and adolescents with Prader-Willi syndrome. *North American Journal of Medical Sciences,* 7**,** 509.

GRIGGS, J. L., SU, X. Q. & MATHAI, M. L. 2013, The Appetite and Behavioural Effect of the Indian Cactus Succulent *Caralluma fimbriata* extract (CFE) on the Markers of Hyperphagia, 2nd Asia Pacific PWS Conference, Sydney, Australia.

GRIGGS, J. L., 2014, Funding Education from a Model of Potential, Disability Studies in Education Conference. Victoria University, Melb. Aust.

GRIGGS, J. L., SU, X. Q. & MATHAI, M. L. 2015d, The Appetite and Behavioural Effect of the Indian Cactus Succulent *Caralluma fimbriata* extract (CFE) on the Markers of Hyperphagia, 3rd Asia Pacific PWS Conference, Melb. Aust.

GRIGGS, J. L., SINNAYAH, P. & MATHAI, M. L. 2015e, Prader–Willi syndrome: From genetics to behaviour, with special focus on appetite treatments. Poster Pres., 3rd Asia Pacific PWS Conference Melb. Aust.

GRIGGS, J. L., SU, X. Q. & MATHAI, M. L. 2015f, Presentation: The Appetite and Behavioural Effect of the Indian Cactus Succulent *Caralluma fimbriata* extract (CFE) on the Markers of Hyperphagia, The Obesity Consortium Conference - Baker IDI.

IV. Prologue

My daughter Mia has Prader-Labhart-Willi syndrome. Prader – Willi syndrome (PWS) is a blend of the names of two of the three men who described the basic presentation of this syndrome in 1956, Andrea Prader (1919–2001) & Heinrich Willi (1900–1971). The third Alexis Labhart (1916 – 1994) is silenced. I understand how unusual a joke is at the start of

scientific thesis. In fact, a prologue is unusual, yet instinctively I feel a need to inform you the reader of the unique complexity within this thesis.

Generally, if you have heard of the syndrome, the name Prader-Willi will conjure in the mind the image of an obese hungry child, most probably standing in a locked kitchen. Yet the image I have of my daughter – who has been diagnosed with PWS - is of a beautiful blonde eleven year old; able to walk her way along the windows of Brunetti's cake counter. She is even able to discuss the elaborate cake designs with just a glimpse of the thought of ordering one. Extraordinary as this seems, it is the reason for this thesis.

Typically, the future for the individual with PWS is portrayed with no chance for independence. At the time of Mia's diagnosis at 18 months, we were told there was no cure, no treatment. The excessive appetite was to be expected and Mia's weight gain would most likely continue. Within this framework my research began. The question we first asked was: "How do we cure Mia's hunger? Luckily this simple question led to an anecdotal victory, which I was determined to research further and share.

Caralluma fimbriata – an Indian cactus succulent, is the protagonist of this PhD research and it is also my daughter's daily breakfast juice. In 2006 (2 years of age) we tried *Caralluma fimbriata* extract (CFE) on my daughter's genuine hunger. Within a short period, Mia's appetite had been successfully attenuated. This intervention was something I wanted to share with the community. Unfortunately, there had been no research on CFE in PWS or in many people at all for that matter, back then. I therefore needed to find out more and though my daughter took no part in this research Mia's early years are an important case study of CFE in PWS (Appendix M).

Unusually, this thesis is informed by my personal journey and daily life with this syndrome. My personal advocacy also instructs the way I write about the "individuals with PWS". Though this is a scientific thesis you may notice I take care to not objectify those with the syndrome. PWS is globally impacting and it has a demanding presentation within a joyous resilient community, of which I am one. The daily battle with the complexities (outlined in the body of this thesis) are individualized and often very overwhelming. Not everyone with

PWS has every component of the syndrome but there is no severity range. You have PWS or you don't. Also the syndrome is not clearly defined by typical disability criteria of "impairment within one category alone (Australian Government funding guidelines). PWS crosses the range of intellectual, physical, sensory and behavioural developmental criteria. The syndrome penetrates all aspects of an individual's development and family life. It also presents researchers with an ever-increasing list of questions and families with a heavy burden for advocacy.

I chose to concentrate my research specifically on what I believe to be most impactful issue of PWS; the "hyperphagia". The increasing hunger and concentration on food is the most sensationalized and frightening aspect of PWS. Any diagnosis of a disability incorporates a devastating internet search for the new parent. In fact, if you place PWS into your search engine and push images you will quickly understand. This vision - an obese adult (often eating cake) is devastating to any new parent of a child with PWS. Altering the hyperphagia is paramount to creating belief in independence for those with this syndrome.

The significance of this original question has led to many long term gains at home for Mia and a new hope for other families throughout the world. Further it has excited an understanding of my pathway and more questions for future research. Though PWS is still pervasive and complex, Mia's appetite is under control and quite simply we have a small Indian succulent to thank for that.

V. Abstract

Prader-Willi syndrome (PWS) is defined by simultaneously non-functioning genes on the paternal chromosome in the critical region 15q11.2–q13, which includes five small nucleolar RNA (SnoRNA), one of which is of interest to this thesis: SnoRNA116/HBII-85. The syndrome has a prevalence of 1:15,000 – 1: 30,000 and the complex physical, behavioural and intellectual difficulties are characterized through four phenotypic phases that correlate with age. Though these phases are individualized in timing and severity, all with PWS will

experience phase three: hyperphagia (mean: - 8 years of age) and will need multiple, life-long intervention programs against the prediction of obesity.

The interest within this thesis has been in reducing the severity, drive and behaviours associated with the hyperphagia in PWS. Examples of these behaviours are delayed satiety, food seeking, repetitive communication, tantrums, hoarding or over-eating. To date there have been no pharmacological treatments or natural supplements to attenuate these appetite difficulties and PWS management guidelines only discuss appetite interventions of diet, exercise or consistent parent/carer meal supervision. Often familial limit setting leads to external food surveillance and locked environments. Antiobesity agents are not advised in any of the scientific literature.

The mechanisms for the PWS hyperphagia have not been fully elucidated, though endocrine irregularities and the brain's neuronal appetite, satiety and reward pathways are implicated. Though there has been much research on appetite-regulating hormones, including leptin, PYY and ghrelin, investigators have not been able to identify the role of serotonin (5Hydroxytryptamine; 5-HT) in PWS. Animal characterization in the literature proposes the loss of the SnoRNA, *Snord116* to be the most likely candidate pertaining to the severity of the syndrome in humans. This genetic microdeletion has been associated with an interruption to the modulation of serotonin signalling in PWS and the *Snord116* deletion mouse model exhibits a similar hyperphagic phenotype to humans with PWS. Selective serotonin reuptake inhibiters (SSRIs) have been proposed to be clinically efficacious in PWS treatment, but research is limited. This thesis determined if a natural supplement, the Indian succulent plant, *Caralluma fimbriata* extract (CFE), was capable of reducing the genuine hunger in PWS.

This hypothesis was due anecdotal evidence of appetite attenuation in a single case study and CFE's reported SSRI-like activity by the proposed active ingredient, the pregnane glycosides.

To this aim the first investigation involved a placebo-controlled, double blind, randomized cross-over trial over a ten-week period to investigate the behavioural markers of appetite in a cohort of children and adolescents with confirmed PWS (n=15, mean age 9.27 ± 3.16 yrs., body weight 43.98 ± 23.99kg). Australia and New Zealand participants ingested CFE or a placebo (PLAC) of maltodextrin/cabbage leaf, over a four-week period, with a two-week

wash-out, before the cross-over to the other treatment. Weekly comparisons in appetite behaviour, severity and drive were recorded by parents.

CFE induced a significant accumulative easing of hyperphagia in one third of the participants, especially at the highest dose of CFE 1000mg/d (recommended adult dose), with a significant decrease in category of appetite behaviour. There were no reported adverse effects at any dose.

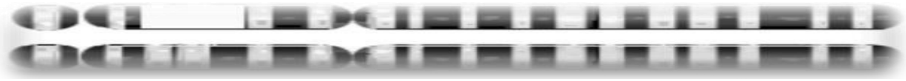
The 5-HT2c receptor enhances appetite suppression. Further, serotonin signalling is implicated in many of the core issues in PWS. The literature describes the HB-II82/SnoRNA116 deletion to be involved in the disruption of serotonin signalling within the hypothalamus. The *Snord116del* (SNO) PWS knock-out mouse model is reported to be a reliable genetically modified animal representative of the of hyperphagia seen in PWS. Therefore, the next study determined if chronic administration of CFE altered the food intake and body weight gain in the SNO mouse model compared to the C57BL/6 wild type (WT) control. This study utilized an eight-week chronic study of CFE and a series of experiments to determine food and fluid intake under basal and stimulated conditions, using a range of acute stimuli, including by glucoprivic and lipoprivic signalling and a serotonin signalling 5-HT2c receptor (SB 242804) antagonist, and 50% food deprivation over four days. All appetite, body weight, appetite signalling and collection of faeces for analysis were carried out in the same animals (total = 72).

The result of study two by a Two-way MANOVA, repeated measure ANOVAs (for univariate significance) and then Post Hoc paired t-tests, determined CFE at two doses (33 & 100mg/k/d) had no significant effect on appetite over the eight-week chronic appetite experiment against the placebo maltodextrin/cabbage leaf (PLAC). CFE did show efficacy in animals with a complex interplay between food appetite, thirst appetite, strain and treatment, under stimulated conditions. Significant differences were mainly observed during 2-deoxyglucose administration (2DG) in glucoprivic feeding and in modulating appetite through the 5-HT2c receptor. Faecal energy content demonstrated malabsorption of chow in the WT controls on the highest dose of CFE (100mg/k/d) and there were very significant differences between strain in the modulation of thirst.

Study three defined if the differences experienced by the animals involved in the behavioural stimulant studies were confirmed by neurological immunohistochemistry after transcardial perfusions. The main interest was to determine if known appetite pathways were activated in the CNS of the same SNO and WT mouse brains after acute glucose deprivation. To do this triple-labelled immunohistochemistry detected c-Fos in cell nuclei and fluorescent co-localization of orexigenic neuropeptide-Y (NPY) activity and the inhibitory neurons of alpha - melanocortin stimulating hormone (α-MSH) in the hypothalamus for appetite and thirst - counted for statistical significance. These results confirmed the behavioural study results with some accuracy and they also demonstrated that further study on appetite and thirst in this model under stimulated conditions was warranted.

In conclusion this thesis determines that an extract of the Indian cactus succulent *Caralluma fimbriata*, eases hyperphagic appetite behaviour within the initial cohort of children and adolescents (n=15) and in the Snord116 deletion mouse model under stimulated conditions. Importantly the animal's studies confirmed CFE's action as an SSRI, interacting with serotonin with significant appetite modulation through the 5-HT2c receptor.

CHAPTER ONE



CHAPTER ONE

1. Introduction

1.1 Prader-Willi syndrome and treatment *Caralluma fimbriata.*

Prader-Willi syndrome (PWS) results from a deletion in the expression of the paternally derived alleles in the region of 15q11.2 - q13 (classified as the PWS region) which was first described by Andrea Prader, Heinrich Willi and Alexis Labhart in 1956, and has a reported prevalence rate from 1:10,000 – 1:30,000 (Whittington and Holland, 2010, Whittington and Holland, 2011, Pignatti et al., 2013, Tuysuz et al., 2014, Smith et al., 2003). PWS is characterized by endocrine abnormalities and complex physical, behavioural and intellectual difficulties. The fundamental issues of growth hormone deficiency, raised ghrelin levels, hyperphagia and a well-defined impaired satiety (Holland, 2008, Miller et al., 2007b), determines PWS to be the most prevalent genetic predictor of obesity (Whittington and Holland, 2010, Holson et al., 2006)

Management of PWS is complex. At its worst the literature documents uncontrolled eating behaviour, "hyperphagia" linked to morbid obesity which may eventuate in associated health problems of cardiovascular disease (Smith et al., 2003) and diabetes type II mellitus (Liu et al., 2010). At its best study sees healthy individuals with PWS on growth hormone replacement treatment (GHRT), with their weight controlled by a community of medical practitioners and carers. Growth hormone (GH) is a peptide hormone which naturally stimulates growth and enables lean mass production in PWS. A technologically produced GHRT is prescribed as best practice in PWS for a variety of reasons and it has been a focus of social and ethical controversies for many years. Unfortunately, those individuals who receive GHRT, still experience hyperphagia (McCandless, 2011).

This predominant point of difficulty remains and therefore so does the issue of behaviour related to appetite control. PWS is a genetic condition where the phenotype follows an evolution - over a number of years - which eventually determines the unremitting hyperphagia. Unfortunately, this is also typically exacerbated by other issues. These include

but are not limited to an impaired satiety (Holland, 2008, Cassidy et al., 2011, Benelam, 2009), obsessive compulsive disorder (OCD) and/or autistic spectrum disorder (ASD)

(McCandless, 2011) and factors which exacerbate the tendency towards obesity. These exacerbating characteristics include a slowed metabolic rate, hypotonic muscle tone, reduced energy expenditure and a concentration on food. Typically, practitioner guidance involves early dietary advice and treatment for the many medical conditions related to the physiology of PWS (Chen et al., 2007, Yearwood et al., 2011).

As mentioned the phenotypical disruptions to appetite follow a specified trajectory seen in figure 1. This also involves transitions in hormone levels which add to the complexity. These include raised ghrelin levels (Del Parigi et al., 2007, Haqq et al., 2003b, Cummings et al., 2002, Holland et al., 2003, Hauffa et al., 2007a, Haqq et al., 2008, Tassone et al., 2007, Purtell et al., 2011), lowered fasting plasma levels of pancreatic polypeptide (PP), raised leptin levels (Goldstone et al., 2012) altered glucose homeostasis (Höybye et al., 2002, Haqq et al., 2007, Goldstone et al., 2001, Goldstone et al., 2012, Druce et al., 2004, Lee et al., 2011), differences in plasma peptide YY (PYY) (Haqq et al., 2003b, Haqq et al., 2007, Purtell et al., 2011), glucagon-like peptide -1 (GLP-1) (Goldstone et al., 2012, Haqq et al., 2011) and cholecystokinin (CCK)(Paik et al., 2007). All these hormones vary relative to age (Haqq et al., 2008) and some influence homeostasis and gastrointestinal motility (Meyer et al., 1989) seen in table 2.

Ghrelin levels are an important regulatory factor of hunger in the general population. It is therefore obvious that in PWS the higher life-long levels must contribute to the hyperphagia in PWS. The efficacy of reducing ghrelin to reduce hunger in PWS is fraught with difficulties. Unexpectedly investigations where treatments are utilized to decrease the serum ghrelin levels in PWS, have not demonstrated a complimentary reduction to the feeling of hunger in individuals with PWS (Holland et al., 2003, Paik et al., 2004). In regards to this unexplained phenomenon the literature documents many disrupted physiological processes in PWS, including disrupted hypothalamic neurology (Holland et al., 2003, Benelam, 2009).

The capacity for the brains pathways to experience appetite signalling is of interest. It is believed the genetic deletion alters the regulation of hypothalamic neurotransmitters and

therefore alters anorexigenic and orexigenic pathways within the hypothalamus (Badman and Flier, 2005). This thesis addresses the question of CFE's neurological action and mechanism for efficacy in interacting with individualized PWS hyperphagia. Importantly the capacity for the supplement CFE to establish efficacy, involves the capacity for the neurology to interact actively in the appetite. This may not be possible in all individuals with PWS as the syndromes neurology may vary genetically. It is therefore important to investigate the regulation of appetite and satiety in both humans and in animals incorporating the genetic variations associated with the hyperphagia.

Also individualized in PWS is the timeline for the characteristics of the PWS appetite behaviour throughout the predicted phased evolution. Though the specific trajectory differs, it is assured that all individuals with PWS will experience hyperphagia eventually (mean age 8 years) and many will be obese (Thomson, 2010, McCandless, 2011). The reason for the timeline differences are unknown but controlled alterations in PWS outcomes are due to administration/or not of growth hormone treatment (GHRT) and the availability of a controlled environments. Therefore, it was important to recruit a group of individuals with PWS exhibiting the typical diversity of PWS symptoms - within a safe range of impairment - utilizing the participants as their own control in a blinded design. Personal PWS symptoms of hyperphagia are most likely well defined by the families/carers of the individual as supervision is most likely established for safety reasons against overeating.

Further the available community of participants with PWS and their parent/carers are not ignorant of the dietary requirements for PWS. All have general practitioner or paediatric care, often through PWS clinics. The community is also very committed to research, knowledge and quality of life. Most acknowledge the need for an appetite treatment within a lifelong timeline. A natural supplement may therefore be better tool for attenuation of the appetite, than a daily injection or clinical procedure.

Also weight-loss and the diet were of minimal interest, due to the need to test the treatment for the use of attenuating hyperphagia within the daily life. There are many different diets for individual with PWS but the main crux is the need to follow a diet of less calories than the general population (approx. 60%) with as much nutrition as possible within the

restriction. Most with PWS will have already been dieting since early childhood. Moreover, weight-loss within a short period is of minimal use. A steady weight is paramount to success in PWS. In the general population a diet for over-weight would be available as an intervention. Yet in PWS diets for weight-loss are very difficult, as the restriction of calories is already in place. When overweight is gained, a diet on top of the diet in place may affect health outcomes. Most likely any further caloric reductions will impede nutritional requirements (Whittington and Holland, 2010).

Due to parameters of weight-loss and hyperphagia in humans with PWS, the behaviours, drive and severity of the appetite are utilized as indicators of appetite control, more-so than weight. Single moments of over-eating are unfortunate and dangerous but they are less representative of hunger than the daily routine. Therefore, behaviours, drive and severity are the research tool of choice over a specific time-line

Once the hyperphagia in PWS is experienced it is often accompanied by temper tantrums (Tunnicliffe et al., 2014a, McCandless, 2011) and anxiety related maladaptive behaviours; for example skin picking (Klabunde et al., 2015). These behaviours while not clearly linked to appetite are demonstrated by a large percentage of children, adolescents and adults with PWS. For example, the temper tantrums will often be demonstrated when hungry and food is not available. Therefore, the need to find food necessitates preventative measures. Further if food is accessible, arresting the caloric intake in a person experiencing a genuine hunger and living with developmental delay or intellectual disability; is very problematic. Due to this the literature does not really explore a capacity for full independence (Goldstone et al., 2008a, Dykens et al., 1996).

It is proposed that early familial supervision of the proximity and choice of food may decrease the consistent preoccupation with food. Families are uniformly the axis of support and restriction (Russell and Oliver, 2003, Goldstone et al., 2008b, Goldstone et al., 2008a) at first and this then extends to the wider community, most likely leading to shared carer facilities after 18 years of age. For some individuals the regulation of the surroundings - which includes locked food storage and/or restricted access in certain environments (McCandless, 2011, Cassidy and Driscoll, 2009) can be considered comforting (Forster and

Gourash, 2005). This is mainly due to food seeking or foraging behaviour (Chen et al., 2007). Continued opportunities for overeating may progress to morbid obesity or in some (especially where the individual has slimmed down) binging may lead to gastric rupture or narcosis (Stevenson et al., 2007).

Unfortunately, practitioner guidance only involves early dietary advice and treatment for the many medical conditions related to the physiology of PWS (Chen et al., 2007, Yearwood et al., 2011). Growth hormone (GH) is the most researched and clearly defined intervention within the PWS community. Unfortunately, GHRT does not impact on appetite. Research into treatments to attenuate the hyperphagia in PWS is imperative to alter the trajectory of the condition.

In regards to appetite treatments in PWS, modification of appetite through pharmacological treatment is often focused on arresting the consequences of the appetite behaviour after the PWS trajectory has caused obesity. There is no precautionary supplementation or long-term modification and pharmacological treatments are limited or non-existent. It therefore became an aim to research CFE's interaction with appetite within the earlier timeline of the aetiology in PWS, opening a path to further research on CFE as an early intervention.

Research into PWS genotype is in its infancy. The PWS-related transitions in hormone levels and the disrupted hypothalamic pathways of the brain do however demonstrate a genomic connection and are important factors in the appetite behaviours. Genes within the critical PWS region correlate to different phenotypic features of the full genetic syndrome which are more often than not documented through animal model research. Atypical micro deletions are found and these have also uncovered some genotypic variation.

There are three distinct genotypes within the PWS "critical region" of chromosome 15q11-13: "deletion or non-inherited" (approx. 70%), uniparental disomy" (UPD) (approx. 25%) - a deletion of the paternal critical region with an inheritance of two silenced maternal alleles of these genes and lastly a small percentage of individuals with imprinting errors" or "micro deletions", which render the paternal contribution non-functional. The paternal contribution is of importance as the maternally derived genes are silenced due to structural modification or epigenetic modulation (DNA methylation) (Dimitropoulos et al., 2000). The above

differences in genotype may define discrete yet significant neurological differences (Yamada et al., 2006, Miller et al., 2007b, Hinton et al., 2007), yet, due to endocrine irregularities all individuals demonstrating the core PWS diagnostic criteria will need restricted ingestion of food.

It seems simple to limit the access to food but the behavioural profile of individuals with PWS lists many difficulties which may impede this intervention. These include issues with learning (Goldstone et al., 2008a), conceptual understanding (Dykens and Shah, 2003), attention switching (Zipf et al., 1990), skin picking, hoarding, re-doing, extensive repetitive questioning (Dykens and Shah, 2003), anxiety, stubbornness and/or temper tantrums (McCandless, 2011). Many of the PWS behaviours are components of ASD and/or OCD (Cassidy and Driscoll, 2009).

Historically, studies on possible pharmacological interventions for appetite regulation in PWS have been limited with individualized and often non-effective outcomes. However, as research into treatments for obesity becomes mainstream, this rare condition may both benefit from and inform pharmacological treatment trials. This thesis outlines the appetite treatments researched in PWS to date (section 2.4 & tables 3 & 4). Unfortunately, the evidence suggests endocrine irregularities in PWS may disrupt the efficacy of pharmacological interventions. Positive outcomes experienced by the general population are not often similarly observed in PWS.

The more recent pharmacological treatment trials in both obesity research and in PWS appetite treatment gives some hope to families. Unusually the adverse effects of some medications for appetite control trialled in the general population are not experienced in PWS. Our research suggests there are positive pharmacological outcomes in PWS but these are more individualized in nature. Therefore, research which determines individual circumstances, genotypes or the mechanisms that alter the hyperphagia in PWS are important.

The objective of investigating an intervention to support food-related abstinence in PWS is very important, as is the individualized responses of easing, decrease or increase in the exhibited hyperphagic behaviours, however further to the documentation of any efficacy in treatment the most important need is the isolating of a mechanism for the effective activity.

Therefore, these studies explore and explain with some precision and complexity, the genetic, endocrine and neurological mechanisms of the outcomes established through three studies. The intervention - determined by anecdotal efficacy- was a natural supplement, *Caralluma fimbriata* extract (CFE). This cacti-form succulent is a hardy roadside shrub well known in Ayurvedic medicine for its attribute of hunger control. This traditional vegetable substitute has been ingested for centuries amongst tribal populations in India (Kuriyan et al., 2007). Though bitter to taste, CFE powder is easy to drink and its safety and toxicity profile has been rigorously studied (Odendaal et al., 2013). CFE is commercially available in many countries, including Australia (Aust.) and New Zealand (N.Z.) where our participants resided.

The investigations of CFE included a four-week oral ingestion of CFE, in children and adolescents with confirmed PWS, within a ten-week cross-over study and a rigorous study of CFE ingestion in a mouse model with a genetic deletion from the critical PWS region. The deletion of the gene *HBII-85 or snoRNA116* in humans has been able to be recapitulated in a mouse model through knock out (KO) of mbii-85 or *Snord116* by the Garvan Institute. At this time this mouse model is utilized as an animal representation of the hyperphagic profile seen in humans with PWS (Herzog, 2012).

There have still only been minimal controlled studies on the ingestion of CFE. The first studies in general were in 2006. Earlier studies determined CFE was able to affect appetite suppression (Kuriyan et al., 2007) and enable significant waist circumference reduction in overweight/obese individuals (Astell et al., 2013). Animal studies demonstrated dose responsive appetite suppression, significant nootropic (improves cognition) effects and anxiolytic (alleviates anxiety) effects in rats (Rajendran et al., 2010).

In regards to toxicology the powdered extract has been investigated at extremely high doses

(5000mg/kg bw/d) and over a long treatment period, with no significant toxicological effect (Odendaal et al., 2013, Rajendran et al., 2010). The earlier pilot study - to this thesis - on CFE, documented a small trend of efficacy (n=5). Even though this was a very small population of children or adolescents with PWS any reduction in appetite is noteworthy in PWS and this favourable reaction warranted further study.

At this time there was minimal literature on the ingestion of the Indian cactus succulent CFE as an antiobesity agent. Due to the anecdotal evidence Victoria University supported the interest with studies on CFE in obese overweight adults (ages 26-60) (Astell et al., 2013) and one study that also supported the investigation for efficacy in PWS was the literature defining an anxiolytic effect in animals (Rajendran et al., 2010). Due to this it was hypothesized that CFE could calm the anxiety experienced in PWS, related to hunger. This thesis has identified some contention to this hypothesis. This may have been the case in the human clinical study documented within this thesis but unusually the opposite was observed in the animal studies.

The treatment CFE is generally known as "Slimaluma®", which is a standardised concentrate of CFE, patented by Gencor Pacific, Inc. (Gencor Pacific Inc, 2007) (http://www.freepatentsonline.com/y2005/0202103.html). The patent claims it is a natural selective serotonin re-uptake inhibitor (SSRI), asserting the pregnane glycosides are the inhibitory factor on the hunger receptors in the hypothalamus. Whether CFE exerts its effect through the influence of serotonergic pathways was one of the objectives for this study. Significantly the results to the appetite signalling study suggest that this is possibly a mechanism for the activity of hunger control.

The neuropeptide serotonin (5-hydroxytryptamine (5-HT)) is a modulator of energy balance and it is known to control the stimulation and regulation of cognitive function, learning, mood, sleep and appetite. These are all targets for intervention within the PWS syndrome's complexity. Indeed, while SSRIs have proven clinically efficacious in the treatment of conditions such as depression and anxiety disorders, their use has also been minimally linked to inhibition of hyperphagic behavioural compulsions in those with PWS (Dykens and Shah,

2003) see tables 3 & 4. The reason for the minimal clinical research on SSRI's in PWS may be due to two reasons. One: an earlier single case study in an adolescent which noted aggressive behaviours due to an SSRI *Fluoxetine* (Kohn et al., 2001) and two: a genetic deletion in PWS which has been established to decrease the efficacy of the 5-HT2c receptor.

To regulate appetite homeostasis, typically 5-HT demonstrates downstream communication within hypothalamic pathways - through the 5-HT2c receptor (Somerville et al., 2007, Fletcher et al., 2010, Schellekens et al., 2015, Lam et al., 2007, Cowley, 2003, Cedraz-Mercez et al., 2005, Xu et al., 2008, Gautron et al., 2015). Disruptions are both known and expected in PWS serotonin signalling. Pathways related to serotonin are important research targets to correct the appetite behaviours in PWS. These include but are not limited to; the 5-HT2c receptor's regulation, through downstream inhibitory pathways i.e. proopiomelanocortin (POMC) – alpha - melanocyte-stimulating hormone **(**α-MSH), and reward targets i.e. dopamine anticipatory appetite.

To define a clearer understanding of the genetic interactions which cause the appetite phenotype and regulation through these targets, researchers utilize atypical micro deletions and KO animal models. Research on deleted genes within the PWS critical region informs correlation for different phenotypic features - of the full genetic syndrome. This makes it possible to utilize KO animals to research specific treatments. Though the results create evidence only in animals, the genomic connections can be linked for further research in humans. Therefore, this thesis investigates the capacity for CFE to alter the hyperphagia in a PWS animal model alongside our human clinical trial (Chapter 5).

I.2 Methodology of Study One, Two and Three

Study One: Clinical trial on the satiety effects of Caralluma fimbriata extract.

Study one is a ten-week double blind, randomized, cross-over trial, where children and adolescents from Australia and New Zealand, with confirmed PWS (5 – 17yrs) (n=16), ingest CFE against the placebo of maltodextrin/cabbage-leaf. This human clinical trial examined the effect of CFE on the hyperphagia and the associated behaviours in PWS over repeated measures recorded by the parent/carers in questionnaires validated for PWS (Dykens, 2007).

Study Two: Animal study investigating Caralluma fimbriata supplementation on appetite and body weight with appetite stimuli tests.

Study two investigated the efficacy of the natural appetite suppressant CFE in altering food intake in animals. The genetically modified (GM) Garvan PWS Snord116 germline (Skryabin et al., 2007, Herzog, 2012, Zhang et al., 2012, Zieba et al., 2015) was paired against a control WT. The efficacy of CFE was tested on appetite in the mice from the expected onset of hyperphagia. Mice received a sham or one of two CFE doses (33mg/kg or 100mg/kg) daily, over an 8-week period. Food intake and body weight were measured daily.

This study also utilizes a complex group of experiments to characterize and establish alterations of appetite and body weight under conditions of altered appetite stimuli. Measurements were taken after stimulated feeding through blocking glucose utilization [2-deoxy-glucose (2DG)], fatty acid β-oxidation [β-mercaptoacetate (MA)], by saline as a control and by food restriction (50% fasting). Lastly this study addresses the suspected PWS dysfunction in serotonin signalling via the 5-HT2c receptor (Benelam, 2009, Holland et al., 2003) utilizing an antagonist to target the 5-HT2c receptor (SB 242084).

Study three: Neuronal representations of PWS; CFE appetite pathways

This neurological immunohistochemistry mechanistic study followed ten weeks of chronic administration of CFE or a placebo (PLAC). Study three verifies if the behavioural experimentation observed in study two in the animals is recapitulated in the neuronal activity expressed through known anorexigenic and orexigenic pathways. Investigations observe and count differentiation in neural cells by comparing activity in the CNS, following perfusion,

ninety minutes after administration of acute glucose deprivation stimuli 2DG in the SNO strain and WT animals.

To do this the mice were deeply anesthetized with an IP injection of sodium pentobarbitone and perfused transcardially by saline and paraformaldehyde in a phosphate buffer. After harvesting the brains, cryoprotection and storage, activated neurons in the mouse brains were observed by confocal microscopy, by immunostaining of the enhanced primary antibody cFos in cell nuclei and co-localization of secondary fluorescence, NPY and □-MSH neurotransmitters in coronal sections of the mouse brain's ARC and PVN of the hypothalamus to detect alterations in appetite and the MnPO in regards to water intake.

The results of these studies determine CFE to significantly alter the appetite behaviour in PWS and demonstrate a need for further studies in both adults with PWS and animal models. representing genetic deletions related to serotonin mediation in PWS.

CHAPTER TWO

CHAPTER TWO

2. Literature reviews

2.1 Prader-Willi syndrome

2.1.1 The genetics of Prader-Willi syndrome

The human genomic locus classified as the Prader-Willi syndrome critical region 15q11–q13 is determined due to a genetic mutation. Atypical expression in the area of chromosome 15, is confirmed through DNA methylation analysis, followed by cytogenetic testing, fluorescence in situ hybridization and microsatellite marker analysis to define the less common genotype classifications (Morin et al., 2011, Rocha and Paiva, 2014). PWS is a contiguous gene syndrome with many simultaneously non-functioning genes on the paternal chromosome 15. These consist of *MKRN3, MAGEL2, Necdin (NDN), NPAP1, SNURFSNRPN* and 5 small nucleolar RNA (*HBII-436/13,438A, 85, 52, 438B*), figure 1. The paternal contribution is important due to epigenetic modulation and the inactivation of maternally derived genes (Dimitropoulos et al., 2000, Angulo et al., 2015).

Deletions in the PWS critical region (figure 1), results in three separate neurodevelopmental disorders: PWS, Angelman syndrome (AS) and 15q duplication syndrome (Chamberlain and Lalande, 2010). AS has similar early feeding problems and developmental delay, sleep disorder, strabismus, hypopigmentation of the skin and similar seizures to some individuals with PWS (Williams, 1995). Seizures are also common in 15q duplication syndromes, medicated by benzodiazepines and ASD is noted (Martin and Ledbetter, 2007, Conant et al., 2014).

The three reported PWS classifications are deletion, UPD or imprinting error or micro deletion (Table 1). These genotypes and common deletion breakpoints, subtypes TI & TII (Munce et al., 2010) define some commonality eg. skin picking is more prevalent in the T1 deletion (Angulo et al., 2015). Other genetic cases may present an opportunity to research distinct variances (Bittel et al., 2006) like a recently presented single case study of a PW-like phenotype, due to duplications on chromosome 6 and 10 (Desch et al., 2015). There are also documented atypical micro deletions within the region 15q11.2-q13.3 (Kim et al., 2012)

which correlate to distinct PWS phenotypic features such as appetite behaviour, IQ and temper outbursts.

Table 1. Current genomic definitions of Prader-Willi syndrome.

Definition	Expression	Prevalence
Chromosome 15 "Deletion" or "Non inherited"	Loss of 3 to 4 megabases of paternal genetic material	(Approx. 70%)
Chromosome 15 "Uniparental disomy"(UPD)	Loss of paternal chromosome 2 maternal chromosomes	(Approx. 20-25%)
Chromosome 15 "Imprinting error"	Error during imprinting, rendering the paternal contribution non-functional.	(Approx. 2-5%)

Table 1 depicts the current genomic Prader-Willi syndrome (PWS) definitions, their expression within the critical region and the percentage of prevalence shown within the general population.

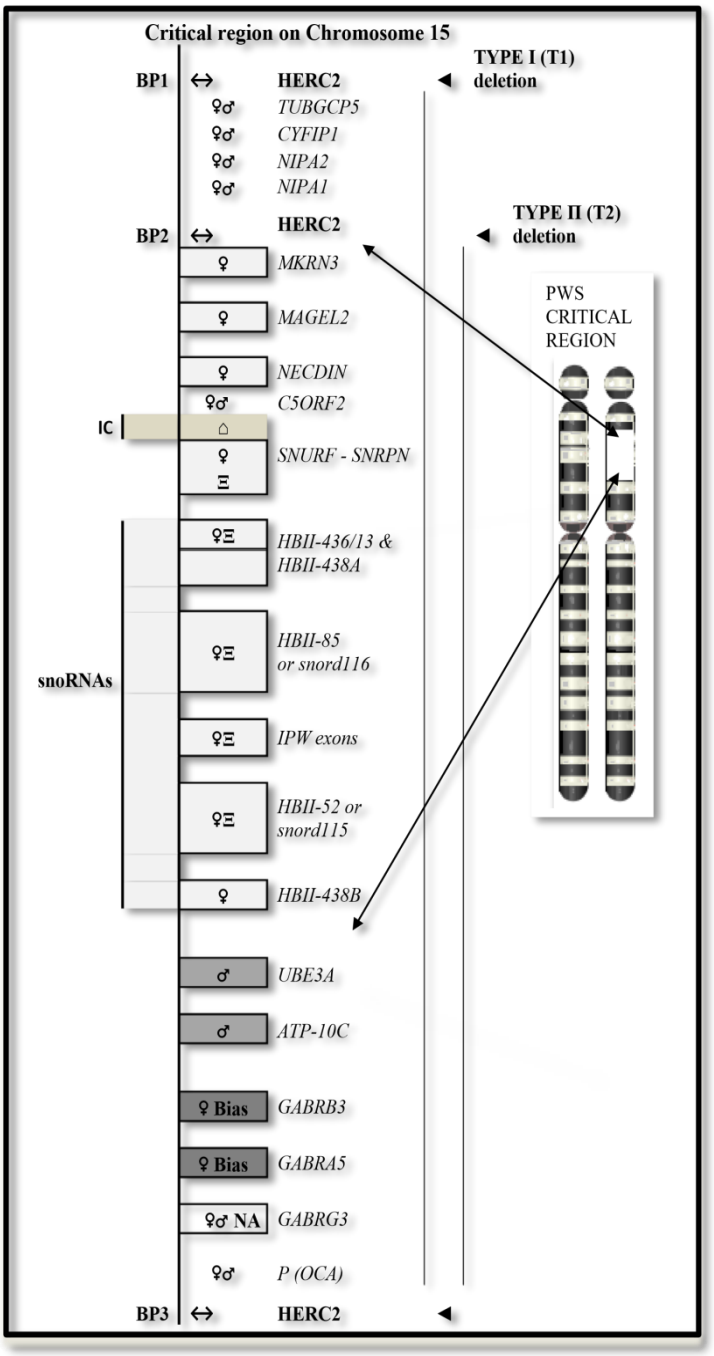


Figure 1. Prader-Willi syndrome Critical Region

Within the human imprinting map of chromosome 15q11.2 -q13.3 is the PWS critical region CEN – centromere, TEL – telomere, IC - Imprinting centre: also involves the Angelman syndrome (AS) ASIC. The PWS-IC is active on the paternal chromosome, yet methylated and repressed on the maternal. BP - break point, TI and II – paternal deletion type one and two, snoRNA - small nucleolar RNA.

The paternal contribution is important due to inactivation of maternally derived genes as a consequence of epigenetic modulation. The DNA methylation utilizes a structural modification involving an additional methyl group, which inactivates genes not required in the cell (Dimitropoulos et al., 2000). Within the PWS genotypes there are common breakpoints due to segmental low copy repeats (LCR) (Munce et al., 2010), which establish subtypes within the deletion status. These subtypes type I (TI) and type II (TII) present an opportunity to research distinct genetic variances (Bittel et al., 2006) as the TI deletion is approximately 500kb larger than the TII (Dykens et al., 2011). The paternal contribution in PWS is believed to be important because the maternally derived genes are inactivated (silenced), due to structural modification or epigenetic modulation (Dimitropoulos et al., 2000). The deleted expression of the paternally derived alleles also has connection to certain neurodevelopmental disorders regarding autism and psychiatric illness (Relkovic and Isles, 2011). Parental specific genetic modifications in this region is classified in PWS by three genotype distinctions.

2.1.2 Genomic methylation in Prader-Willi syndrome

Methylation of DNA is a transcriptional silencing which determines the sequence specific repression of certain genes. In humans, the genes of chromosome 15q11 - q13 are exclusively paternally expressed, therefore this involves DNA methylation where transcriptional silencing takes place in retarding the expression of maternal genes. The methylation mechanism in multicellular eukaryotes involves a targeting of utilizing DNA methyltransferase enzymes. This results in histone modification and chromatin remodelling (Klose and Bird, 2006). Methylation sensitive enzymes have been studied in PWS and AS research, with the hope of discovering the underlying imprinting mechanisms. It is believed that the SNRP gene corresponds with the parent-of-origin expression and that this gene takes on an important role in imprinting during gametogenesis (Zeschnigk et al., 1997). The other simultaneously non-functioning deleted genes within the PWS critical region functionally alter developmental processes but it is not clear how or if these genes communicate with each other or whether knocking out the genes disrupts any of the silencing related to proximal genes or even other chromosomes.

2.1.3 Animal models in Prader-Willi syndrome

Gene knockout models have been utilized to uncover elements of the genetic loci associated with PWS appetite behaviours (Ding et al., 2008). Research has uncovered some of the attributes of certain genes. The human PWS critical region is represented by the mouse chromosome 7C region (Kantor et al., 2006). Deletions of G protein coding genes, *Necdin* (Ndn) and *Magel2* are implicated in disturbances early axonal growth (Lee et al., 2005) and *MKRN3* is implicated in early pubertal development (Abreu et al., 2015). More recently the function of paternal Grb10 has been associated to behavioural influences of temper and social dominance which is interesting in regards to the behavioural experience in phase three of the PWS appetite behaviour (Isles, 2015). Unfortunately, animal models do not completely replicate the human expression of PWS and some PWS representational animal models have low viability (Herzog, 2012).

Expression of *Magel2* in neurons, is most prominent during the development of the hypothalamus and is implicated in cytoskeletal organization, whereas *Ndn* is implicated in the development of the nervous system. *Magel2* null mice pups have a slowed growth rate, circadian rhythm disruptions and altered food intake and the adults show increased adiposity (Mercer et al., 2013). *Magel2* null mice have demonstrated a loss of depolarization in neuronal responses to leptin within the brain's arcuate nucleus (Mercer et al., 2013) - an area associated with appetite. Importantly, these mice experience catch-up weight gain after weaning, which may demonstrate this gene's involvement in the human PWS phase two, yet *Magel2* targeted mutation models are hypophagic (Mercer and Wevrick, 2009). This unusual discrepancy could be due to reduced locomotor activity or a lower basal metabolic rate. Also, circadian rhythm irregularities have been shown to induce sleep apnoea (Hilaire et al., 2010), which may disrupt the metabolism.

Ndn null mice embryos show disturbed cortical commissural formation, misrouting of hypothalamic and dorsal thalamus axons and the absence of serotonergic-positive fibres (Lee et al., 2005). Distribution of this gene results in abnormal morphology in medullary serotonin (5-HT) vesicles (Zanella et al., 2008). Treatment strategies may include epigenetic targeting of *Ndn,* to investigate if re-induction of this gene could establish neuronal migration in the *Ndn* null mouse model.

Importantly within the PWS genetic loci, there are disruptions of transcriptions which include SnoRNA genes located on the introns or "intragenic region" (a nucleotide sequence within a gene removed by RNA transcription). SnoRNA *HBII-52* or *SNORD115* and *HBII-85* or *SNORD116* are involved in the alternative splicing of mRNA (Munce et al., 2010, Runte et al., 2005, Sahoo et al., 2008, Duker et al., 2010). This has functional consequences, including influencing serotonergic pathways and circadian rhythms.

The neurotransmitter serotonin or 5-hydroxytryptamine (5-HT) regulatory processes seem to be problematic in PWS as many of the core difficulties involve serotonin regulation within central nervous system (CNS). These include the modulation from the gut to the CNS (O'Mahony et al., 2015), thermogenesis (Schneider and Nadeau, 2015), maladaptive behaviours, sleep and dopamine-related anticipatory appetite (Dimitropoulos et al., 2000, Holland et al., 2003, Lam et al., 2007) and regulation of hypothalamic and pituitary gland hormones (Fulton, 2010).

Whilst the dysfunction of serotonergic signalling is implicated in PWS appetite behaviour, efficacy of SSRI use in PWS is not clearly established (Kohn et al., 2001). The 5-HT2c receptor regulates downstream appetite inhibition through POMC activation of alphamelanocortin signalling via the MC3 and MC4 receptors (Cowley, 2003, Farooqi and O'Rahilly, 2007) (Section 4.3.1 & figure 11). In PWS, SSRIs are utilized for OCD maladaptive behaviours such as skin picking (Soni et al., 2007). To develop the understanding of the disturbed appetite in PWS, further research is needed to evaluate the brains corresponding 5-HT uptake and the downstream pathways for excitation through the α-MSH - in genetically modified animal models (Griggs et al., 2015a). This may inform pharmacological regulatory treatment for human clinical trials. Individualized genotyping may also determine those individuals who could find treatment beneficial. Further, herbal bioenhancers could be incorporated or designed to be available for phamacotherapeutics in PWS; for example, there are many biobehavioural effects of natural products on appetite, mood and cognition (Ravindran and da Silva, 2013).

Though PWS micro RNA (miRNA) encoding research is in its infancy, researchers have determined that both the *Snord116* and *115* have a complex influence during alternative splicing of mRNA coding for 5-HT2c receptors, which are reported to be brain-specific

(Schellekens et al., 2015, Canton et al., 1996). The *Snord115* may play a role in PWS appetite dysfunction as this gene encodes the 5-HT2c gene specifically in the brain (Wylie et al., 2010) (Sections 4.2 & 4.4.3). The PWS-IC +/- mouse model is the representational model for the *Snord115* deletion, yet due to low viability, the function of the *Snord115* pre-RNA editing is difficult to investigate (Kishore and Stamm, 2006, Doe et al., 2009, Morabito et al., 2010).

The *Snord116* is very strongly represented in areas of the brain related to feeding control including the hypothalamic ARC and PVN (Zhang et al., 2012). The *Snord116* (also named *MBII-85*) is a C/D box snoRNA with the sequence: AAT GAT GAT TCC CAG TCA AAC ATT CCT TGG AAA AGC TGA ACA AAA TGA GTG AAA ACT CTG TAC TGC CAC TCT CAT CGG AAC TGA.

This duplex oligonucleotide (short acid polymer) of sense and antisense is held for study at Integrated DNA Technologies (IDT, Inc.)(Zhang et al., 2014). Luckily *Snord116* mouse models with this deletion are not as vulnerable as the *Snord115* deletion model though they are consistently smaller than wild type mice (Duker et al., 2010). It has been noted the brains transcription of this gene is developmentally engaged in the hypothalamus, meaning at different ages there will be more or less of the *Snord116* transcription (Zhang et al., 2014)

This mouse model studied in this thesis's animal treatment study, demonstrates increased hyperphagia after seven weeks (Ding et al., 2008) and up-regulation of neuropeptide Y (NPY) expression levels in the ARC. neurons of the hypothalamic(Qi et al., 2016). *Snord116* null male mice, have increased insulin sensitivity, as opposed to females, but both have agerelated extended bouts of eating, altered metabolism (Ding et al., 2008) and increased respiratory rates relative to intra-abdominal adiposity, leptin and insulin sensitivity (Cnop et al., 2003). Lastly PWS animal model research may inform our understanding in the regulation of ghrelin related to the environment and comfort food (Schellekens et al., 2012, Steculorum et al., 2015).

In conclusion animal models are useful in defining the reciprocity of the genetic loci and for establishing the viability of treatments for single genetic targets. However, they do not represent the full human PWS phenotype for pharmacological evaluation. As yet no animal

model is an adequate representational model for the PWS transition to obesity. Human trials are therefore imperative to our understanding for treatment efficacy in PWS hyperphagia.

2.2 Progressive hyperphagia and behaviours in Prader-Willi syndrome

2.2.1 The hyperphagia in Prader-Willi syndrome

The appetite behaviour in PWS is life threatening. The literature describes families as the axis of support in food restricting routines. Over the trajectory of PWS, appetite issues are confounded by, among other things - late diagnosis, reduced daily caloric intake (Goldstone et al., 2008a), anxiety, learning/cognition difficulties, mood related disorders, temper tantrums and aggressive behaviours (McCandless, 2011, Tunnicliffe et al., 2014a).

In PWS there are four phenotypic phases seen in figure 2, correlate with age (Butler et al., 2010). The core diagnostic criteria for PWS during infancy include: hypotonic muscle tone (described as floppy), observed in 100% at birth (Tuysuz et al., 2014), weak cry, inactivity and feeding difficulties (Holm et al., 1993, Whittington et al., 2002). Later predictors are obesity, eating disturbances, short stature (adults: male <156cm, female <150cm) (Cassidy and Driscoll, 2009), incomplete gonadal maturation and learning disabilities (Goldstone et al., 2008a).

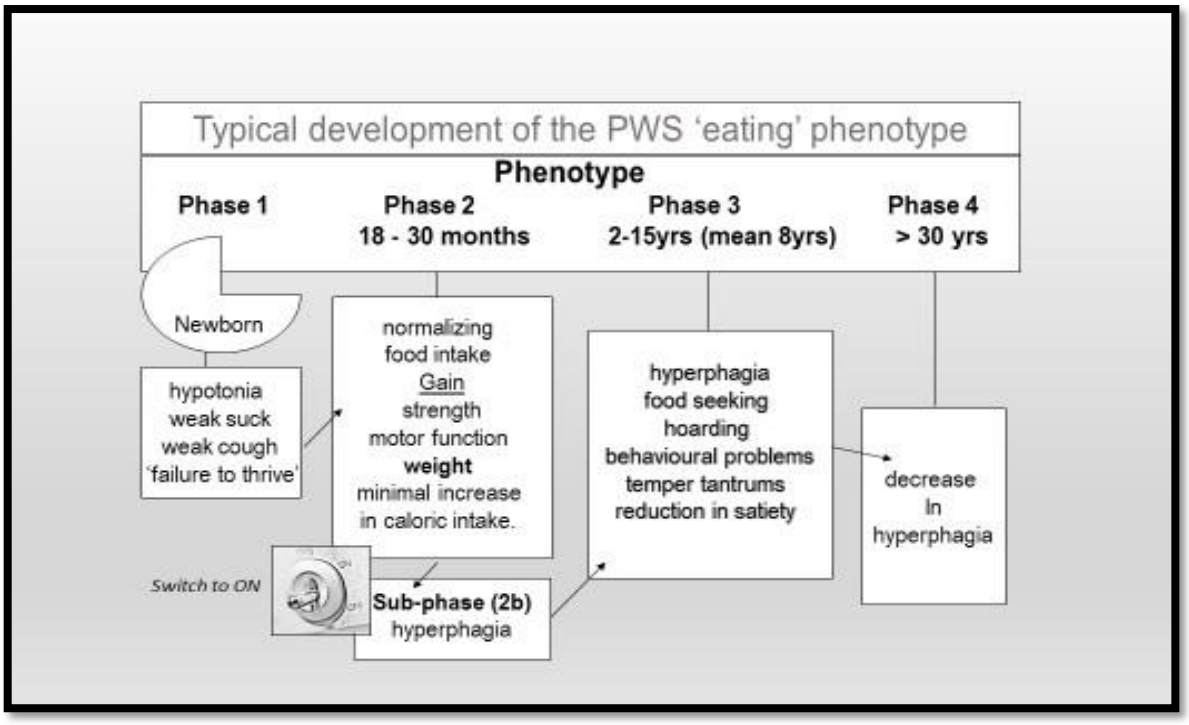


Figure 2. The Prader-Willi syndrome phenotype phases

From phase 1: newborn, through to the switch to hyperphagia and progressing through to phase 4: adults over 30 years, as reported by (McAllister et al., 2011b)

During phase 1: "failure to thrive", the newborn with PWS sleeps more constantly and feeding may be supplemented by naso-gastric tube due to a weak sucking reflex. Maturation leads to an increased arousal (also classified as phase 1b (Salehi et al., 2015). This is followed by phase 2 where developing feeding ability leads to a significant increase in body mass index (BMI) without relative additional caloric intake (15 - 30 months) (Goldstone et al., 2008a, Butler et al., 2010, Goldstone et al., 2012, Miller et al., 2011). There are no clear correlations between hormone levels, early onset weight-gain or the development of the hyperphagia. Gains in physical strength and developmental capability are possible at this time even though the body's hypotonic muscle tone prevails indefinitely (Goldstone et al., 2012). Lifelong, individuals with PWS will exhibit a decreased resting energy expenditure, and a lower lean muscle:fat ratio in comparison to BMI matched subjects.

During phase 2 there is an increased respiratory quotient, indicating a switch toward increased carbohydrate metabolism before the weight gain (Miller, 2012). During sub-phase

2b (after the weight gain), hyperphagic eating issues start to become obvious to parents/carers (median 43 months). Hyperphagia (figure 1) progresses and phase 3 lasts between 2 - 15 years (mean 8 years) (Thomson, 2010, McCandless, 2011), where many individuals with PWS experience extreme appetite behaviours i.e. food seeking and temper tantrums. This phase requires supervision and preventative measures as the hyperphagic behaviours will progress to obesity.

Obesity, in PWS, seems to be independent of caloric intake under five years of age (Goldstone et al., 2012). The phase changes are therefore largely attributed to unknown disturbances in neurohormonal signalling (Hochberg and Hochberg, 2010, McAllister et al., 2011b, Lloret Linares et al., 2013). This thesis clarifies the known contributing factors in section 2.3.

For all diagnosed cases of PWS food related supervision is recommended. Once phase 2b is experienced, the diagnosed individual will need a lifelong commitment by the extended community to create a routine of "no free access" to food (Steinhausen et al., 2004).

Full independence in adulthood is not expected, therefore psychosocial complexities of adult containment cause some debate (Dykens et al., 1996, Goldstone et al., 2008a). Generally, the accepted view is that those with PWS are incapable of making food-related decisions (Russell and Oliver, 2003, Goldstone et al., 2008a) and informed families should regulate weight early (Cassidy and Driscoll, 2009, McCandless, 2011). In many families constraints are installed to deter food seeking behaviour (locked kitchens cupboards and refrigerator) (Chen et al., 2007), sometimes even before hyperphagia related behaviours are noticed.

Recommendations from clinical professionals include dietary advice (Holland et al., 2003) for nutritionally dense foods (Yearwood et al., 2011, Miller, 2012), establishing routines for the timing of meals and suggestions of regular exercise. Other considerations are supplementation of vitamins, iron and calcium (Lindmark et al., 2010), small, frequent portions (6 meals /day) and psychological tools e.g. the illusion of abundance using small plates (Miller, 2012). Recommendations from researchers believe identifying the progression of hormones will be more beneficial in defining the timeline of the phases and the need for intervention (Kweh et al., 2015).

Historically, the body composition in PWS (shortness in stature with hypotonic muscle tone) is a precursor to low post prandial energy expenditure and decreased caloric intake (Purtell et al., 2015). Importantly, new generational "best practice" includes GHRT (McCandless, 2011), which typically increases height and the muscle:fat ratio. Though GHRT may assist physical agility (Bertella et al., 2007) it does not change the appetite in PWS.

Most recently the treatment for hyperphagia (anecdotally preferred by parents) is a behavioural routine called "FOOD SECURITY", (Forster and Gourash, 2005). This is a habitual long term strategy given to parent/carers at conferences for PWS worldwide. The intervention involves controlling the environmental access to food through deliberate repetition of a catch cry with appropriate actions to back these up. The sentence is "no doubt, no hope and no disappointment", (Forster and Gourash, 2005). This means: no doubt, when meals will occur and what will be served, no hope, of getting anything different from what has been planned and no disappointment or false expectations for more food. This intervention is supposed to control the anxiety experienced by an individual with PWS when food is freely available.

Phase 4 (>30 years) is characterized by a reduction in hyperphagia. The origin of this reversal is also unknown. Research has been limited due to the high mortality and obesity-related illnesses, though it is suspected that GHRT will establish more longevity (Dykens, 2013). Unfortunately, Dykens reports that the decreased hyperphagia may not correlate with a reprieve from behavioural distress or depression. This phase includes self-absorbed behaviours, daytime sleepiness and episodes of psychopathology (Sinnema et al., 2012)

The hyperphagia in PWS may progress through these phases within an individualized timeline mainly related to the hormone levels, associated with their own genotype and inherited DNA (Heymsfield et al., 2014). For individuals with PWS there are many other chronic difficulties which may confound the capacity for treatment, including temperature dysregulation, high pain threshold, an inability to vomit, scoliosis, sensory impairment, a risk of seizures, thick saliva and dental problems (McCandless, 2011, Yearwood et al., 2011, USA, 2015). These attributes also warrant observational vigilance by carers and researchers, especially during pharmacological trials tables 3 & 4. Many of the physiological issues mentioned may confound the hyperphagia especially during phase 2b-3 where compulsive

behaviours restrict the individual with PWS's routine, in part due to a delayed satiety (Dykens and Shah, 2003). An example of this was demonstrated in a study on the effect of the opiate antagonist *naloxone* on appetite (Zipf et al., 1990) section 2.4.3. The test demonstrated the extreme hyperphagia in PWS children and unremitting compulsive behaviours especially within the proximity of food. Within this appetite trial, individuals with PWS phase 2b, were presented with continual plates of chicken sandwiches - for over an hour in duration. Participants were given this stimulus of food ad libitum with no capacity to leave the room and the number of consumed sandwich quarters were counted. Most of the PWS participants continued to eat after administration of the treatment and placebo; one subject ate up to 100 sandwich quarters. It was noted that this was mainly due to individualized delayed satiety, demonstrating varied time lines for satiation (Dykens and Shah, 2003).

When fully within phase 3 and PWS adulthood, mood related disorders and aggressive behaviours may become prevalent (McCandless, 2011). At this time the need for control of PWS hyperphagia, satiation and OCD behaviour is paramount to the survival of the individual with PWS, demonstrated well by international PWS community slogan "still hungry for a cure" (USA, 2015). The literature does not really explore a capacity for full psychosocial independence and carer decisions and age related guardianship complexities related to restriction causes some debate (Goldstone et al., 2008b, Dykens et al., 1996). Intellectual ability may impede reasoning when concurrent with hyperphagia and compulsive food seeking. Further reasoning related to food may include temper tantrums (Tunnicliffe et al., 2014b). The accepted view is that families must support the abstinence of the individual with PWS as they thought to be incapable of making food related decisions (Russell and Oliver, 2003, Goldstone et al., 2008b). These means families must use many creative practises to alter behaviour or temper when related to food (Tunnicliffe et al., 2014b) well into adulthood.

2.2.2 Obsessive compulsive disorder, IQ and autistic behaviours.

For all with PWS there will be cognitive learning issues and developmental delay (Goldstone et al., 2008a) and most will present with moodiness, executive function issues (Chevalère et al., 2013) and OCD and/or ASD (Cassidy and Driscoll, 2009, Hogart et al., 2010, Lo et al., 2013). The IQ in individuals with PWS ranges from 40 – 105, though a higher IQ does not predict a better outcome in learning (Dykens and Shah, 2003). Furthermore, individuals' express maladaptive behaviours i.e. skin picking, hoarding, re-doing and repetitive single subject questioning and speech (Dykens and Shah, 2003), and there are many food and non-food related individualized OCD behaviours. Examples may be: organizing the plate to eat each food group separately (Zipf et al., 1990) and uniquely arranging the utensils (Dykens et al., 1996) or repetitive clock watching and zipping up a jacket over and over (Griggs et al., 2015). OCD behaviour related to food and "being allowed to" in PWS is complex. The clarity from the family regarding proximity and choice may eliminate the requirements placed on a person with PWS. It is stated that doubt of "can" or "can't" creates anxiety and temptation, whereas sameness creates will power. Even so obsession will be played out with objects and food. For example, food may be eaten in order of texture, type, colour or desirability and within parameters such as very fast or slow at specific times or only when utensils are arranged in a specific preordained and expected manner (Dykens et al., 1996). It has been found that there is less 'switching of food' (changing food in between bites) than matched controls (Zipf et al., 1990) and that those with PWS may eat food that is contaminated, old or a non-food item (paint or paper) or even pet food (Dimitropoulos et al., 2000). Unfortunately, this detail and complexity is not addressed by the measurements stated within the questions of the PWS hyperphagia questionnaires (Appendix 2) in study one. Even so individual markers of anxiety and OCD have been documented by the parents in journals. These individualized markers may be an interesting research topic in future

Though the symptoms of OCD seem autistic in nature (Dykens et al., 1996, Lo et al., 2013), i.e. presenting as ritualistic, inflexible and repetitive in concern, (Dimitropoulos et al., 2000, Clarke et al., 2002b, Dykens and Shah, 2003) subjects with PWS are less self-absorbed (Dimitropoulos et al., 2009) or more social than the ASD criteria. The diagnosis of ASD in PWS is only 25% (Cassidy and Driscoll, 2009) and treatment includes cognitive behaviour therapy (CBT).

Similar to autism the OCD in PWS is linked to developmental delay (Clarke et al., 2002b) and it is demonstrated by many idiosyncratic concerns. The main mal-adaptive issues are skin picking, hoarding, re-doing, 'asking and telling'(Dykens and Shah, 2003), which do resemble ASD. A study which left out hyperphagic behaviour, compared OCD in PWS with controls (Dykens et al., 1996) noting that statistically significant symptoms of OCD in PWS seem more autistic in nature and that ritualistic behaviours are aimed more at reducing anxiety than increasing enjoyment. Diagnosis of autistic spectrum disorder (ASD) in PWS is 25% (Cassidy and Driscoll, 2009) as many individuals exhibit behaviours that are repetitive and ritualistic such as concerns with symmetry, counting and arranging (Dimitropoulos et al., 2000, Dykens and Shah, 2003, Clarke et al., 2002b). These behaviours may or may not be food related. From school age there is an increased risk of tantrums and aggressive behaviour due to routine alterations in day to day living. As reported later in life, psychosis may be seen, more common to those with the genotype of UPD (table 1.)(Cassidy and Driscoll, 2009, Dykens and Shah, 2003) .

Research into the specific behaviour associated with genotype in PWS is in its infancy, however a propensity for psychotic behaviour is more common to the genotype of UPD (Dykens and Shah, 2003, Soni et al., 2007, Cassidy and Driscoll, 2009). There may also be an increased risk of ASD in the UPD genotype, but this is weakly correlated (Dykens et al., 2004, Hogart et al., 2010, Lo et al., 2013). This thesis does not address pharmacological treatment for psychosis (Soni et al., 2007) but it does touch on maladaptive behaviours related to appetite.

2.3 Aetiology in Prader-Willi syndrome

2.3.1 Prader-Willi Syndrome energy homeostasis

In general, energy homeostasis is linked to a complex regulation of caloric intake and energy expenditure, aligned to compensatory modulation within the system. The known PWS-related transitions in hormone levels are as follows. From 7mths – 5yrs, there are significantly lowered fasting plasma levels of pancreatic polypeptide (PP), while leptin levels are significantly raised (Goldstone et al., 2012). There is conflicting opinion on the correlation between plasma ghrelin levels and the sensation of hunger or caloric intake in PWS. Ghrelin secretion from the stomach is known to modulate appetite and glucose homeostasis, interacting with levels of plasma peptide YY (PYY), leptin, glucagon-like peptide -1 (GLP1) cholecystokinin (CCK) and the anorexigenic and orexigenic pathways within the hypothalamus (Badman and Flier, 2005). Ghrelin levels in PWS are elevated against healthy weight and obese subjects (DelParigi et al., 2002, Haqq et al., 2003b) and they clearly vary relative to age (Haqq et al., 2008), with higher ghrelin levels in older children. CCK levels are also elevated in children (Paik et al., 2007) though adults have an altered responsiveness measured by radio immunoassay in regards to the stimulation induced by rising free fatty acid plasma levels compared to obese controls (Butler et al., 2000). Constipation is prevalent in PWS despite higher levels of ghrelin (Asakawa et al., 2001) and CCK which influence gastrointestinal motility (Meyer et al., 1989).

In children with PWS baseline ghrelin levels are higher (Paik et al., 2006). Further the bioactive form of ghrelin (acylated ghrelin) and total ghrelin differ in regulation during GH administration (Hauffa et al., 2007b). The post prandial reduction in ghrelin has a correlation to the availability of insulin. In individuals with PWS insulin resistance and raised acylated ghrelin levels are observed during the first months of GH administration. This differs from total ghrelin which does drop during glucose administration similar to obese controls (Paik et al., 2006). Interestingly it is proposed that increased ghrelin levels in PWS are comparable to a syndrome of starvation (Holland et al., 2003) similar to anorexia nervosa (Tassone et al., 2007), yet unlike anorexia, when post-prandial or treated, decreased ghrelin levels are not experienced as satiety. This may simply be due to the fact that ghrelin levels still remain higher than in obese controls (Paik et al., 2004).

Similarly, ghrelin normally stimulates GH releasing hormone, yet GH levels in PWS are decreased (Goldstone et al., 2012, Aycan and
Baş, 2014, Bridges, 2014). This may be explained by the continual chronically high ghrelin signals, perhaps leading to a desensitizing of the growth hormone secretagogue receptor (GHS-R) (Holland, 2008). Even so, GHRT does not resolve the hunger experienced in PWS (Goldstone et al., 2012, Deal et al., 2013).

Ghrelin has been found to modulate the sympathetic nervous system (SNS) (Lambert et al., 2011, Schellekens et al., 2013) inducing a drop in blood pressure, stress levels and anxiety in both obese and lean humans. Further, research into ghrelin has demonstrated mood-related properties, with levels in mice significantly elevated by periods of chronic social defeat stress
(CSDS) (Lutter et al., 2008). Ghrelin's orexigenic effect may be modulated by serotonergic processes through 5-hydroxytryptamine (5-HT) receptor: 5-HT2c signalling (Schellekens et al., 2015). Modulation of hunger and energy homeostasis may therefore be mediated by mood regulation (Bays, 2004, Talebizadeh and Butler, 2005) in a two-way interaction (Lutter et al., 2008, Lambert et al., 2011) and perhaps this may provide an opportunity for an intervention through selective serotonin re-uptake inhibitor (SSRI) treatment and CBT.

Appetite and satiation are biological processes utilizing neurological signals sent from various organs and tissues to the hypothalamus, brain stem and higher cortical areas. Figure 3 below, shows the known core signals and regulatory interactions in play (Xu et al., 2008, Heisler et al., 2006, Cowley et al., 2003, Cowley, 2003, Arora, 2006, Sainsbury et al., 2002, Cone, 2005, Woods et al., 1998, Parker and Bloom, 2012), necessary for the homeostasis of energy balance, intake, expenditure and sleep wake cycles (Van Cauter and Knutson, 2008).

Determining the underlying complexities which establish the phenotypical physiology and behaviours of PWS within this homeostasis is difficult due to the complex interplay of behavioural, pathophysiological and psychological processes. Disturbances in hormone levels may be disrupted due to the physiology of PWS and many of these interactions may have disrupted regulation due to unknown hypothalamic disturbances. Even emotional reactiveness can cause further hormonal interference, which will eventually lead to more complexity within the neurology. In this way the genetics of PWS create complex feedback loops of disruption. Table 2 notes the established significant regulation issues in PWS.

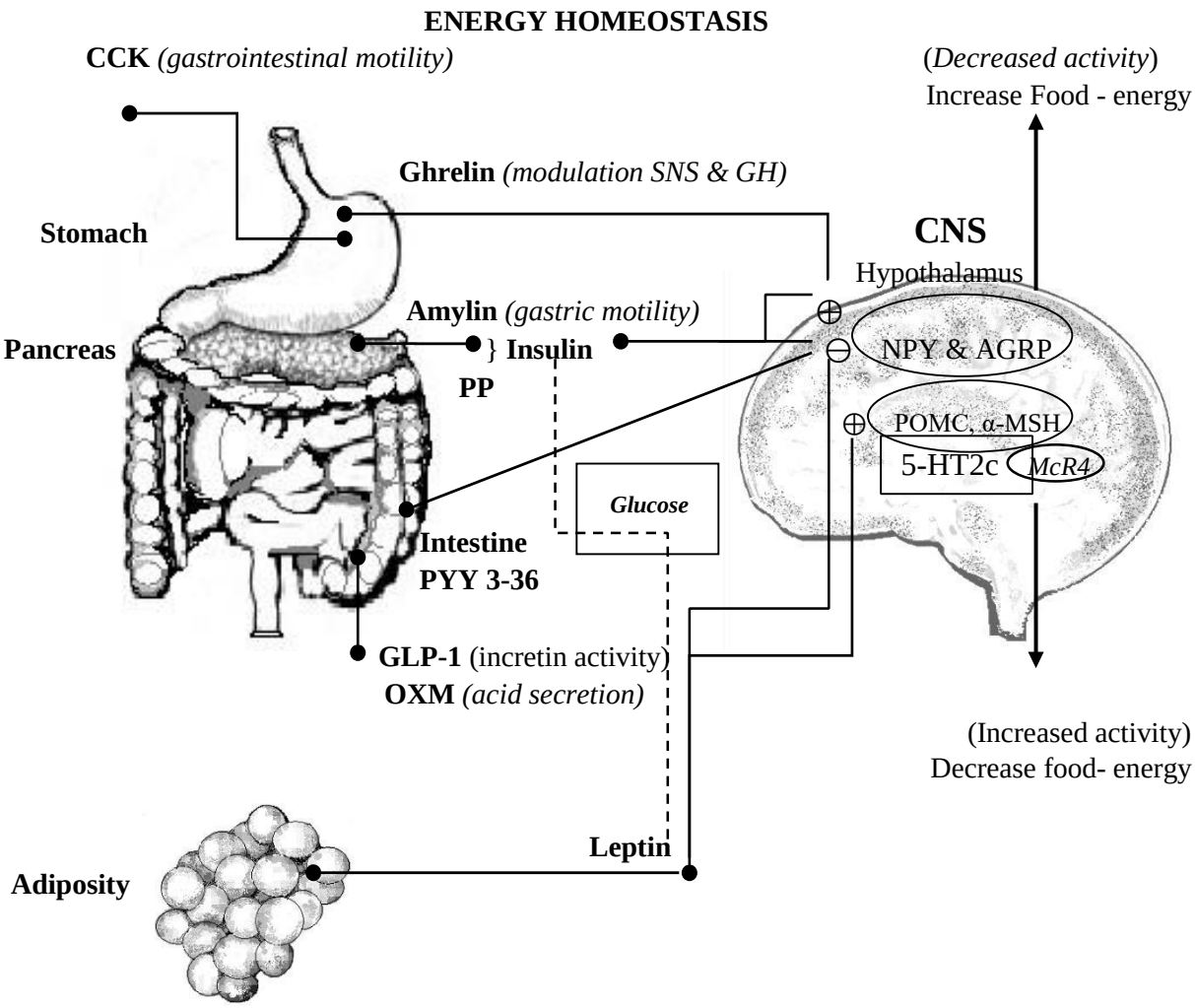


Figure 3. Appetite Interactions within the Body

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Neural interactions of appetite, satiety energy balance (Benelam, 2009) showing the gut, brain and adipose tissue. Ghrelin: hunger and growth hormone release; Leptin and Insulin regulates glucose homeostasis and Amylin interacts in gastric motility. CCK - cholecystokinin: gastrointestinal motility and pancreatic exocrine secretion; PP - pancreatic polypeptide, PYY - peptide YY: involved in satiation; OXM - oxyntomoduli: also satiation and acid secretion; GLP-1 - glucagon-like peptide -1: incretin activity and satiation. Hormonal, neural and psychological processes adapt in combination with our habitual and environmental interactions as energy balance.

Other than ghrelin, fasting insulin levels in PWS are higher in children, with an increased resistance to insulin correlating to age (Höybye et al., 2002, Goldstone et al., 2012). Female adults with PWS have lower fasting insulin, though these levels are similar to obese controls when adjusted for central adiposity (Goldstone et al., 2001). Circulating adiponectin ratios are higher than obese controls (Haqq et al., 2007). Homeostasis model assessment of insulin resistance (HOMA-IR) is significantly lower in PWS children, compared to BMI matched controls (Haqq et al., 2007). However in adolescents, similar plasma insulin/amylin ratios have been measured between pre-pubertal PWS adolescents and obese controls (Lee et al., 2011). This is accompanied by a significant delay in the secretion of amylin after glucose loading in the subjects with PWS. In adults with PWS, baseline values of insulin (Goldstone et al., 2005, Haqq et al., 2011) are reduced before a meal and GLP-1 increases postprandially which causes an increase in the amount of insulin released (Druce et al., 2004). In contrast to the insulin resistance, improved insulin sensitivity is documented in PWS (Talebizadeh and Butler, 2005, Goldstone et al., 2012).

Leptin sends signals from adipose tissue to activate neurons regarding appetite homeostasis by stimulating the expression of orexigenic neuropeptides, neuropeptide Y (NPY), agoutirelated peptide (AgRP) and the anorexigenic cocaine-and amphetamine-regulated transcript (CART) and the precursor peptide pro-opiomelanocortin (POMC) in the arcuate nucleus (ARC) which mediates α - melanocyte-stimulating hormone (α-MSH) of the paraventricular nucleus (PVN) (Cowley et al., 2003) (See Figure 3.)

Table 2 Aetiology in PWS

Interactions for dysregulation of homeostasis in PWS.				
Reference	**Age**	**Hormone**	**Effect & Phase**	**Interaction**
(Goldstone et al., 2012)	7mths – 5yrs	PP	Lower Phase 1 – 2b	Pancreatic exocrine secretion, PP is involved in satiation along with PYY.
(Goldstone et al., 2012)	7mths – 5yrs	Leptin levels	Raised Phase 1 – 2b	Leptin stimulates appetite via activation of hypothalamic NPY and ARGP neurons within the CNS, whilst simultaneously inhibiting appetite through the expression of anorexigenic POMC. Leptin also interacts with insulin for glucose homeostasis.
(Cummings et al., 2002, Holland et al., 2003, Hauffa et al., 2007a, Haqq et al., 2008, Tassone et al., 2007)	Children, adolescents & adults	Ghrelin levels	All raised, higher in older children (phase 3)	Ghrelin modulates hunger, the SNS and growth hormone release. Ghrelin is also regulated by serotonin, creating an interaction between mood and appetite.
(Goldstone et al., 2012, Goldstone et al., 2001, Höybye et al., 2002, Haqq et al., 2007)ce et al., 2004 Lee et al., 2011	Children adolescents & adults	Fasting insulin levels	Raised in children, similar to obese adolescence. Both increased resistance & improved sensitivity over time, lower in female adults	Insulin regulates glucose homeostasis by promoting the uptake of glucose and metabolizing carbohydrates and fats. Insulin interacts by increasing leptin.
Lee et al., 2011	Adolescents	Amylin secretion	Delayed	Amylin interacts in gastric motility
(Goldstone et al., 2012, Haqq et al., 2011)		GLP-1	Lower with postprandial increase.	GLP-1 has a role in satiation by stimulate insulin and decreases glucagon.
(Haqq et al., 2003b, Haqq et al., 2007)	Children & adults	PYY levels	Higher & negatively correlated with age	PYY interacts by inhibiting NPY in the CNS, it also inhibits gastric motility and increases water absorption in the colon.

Table 2 presents the dysregulation factors in Prader-Willi syndrome, PP - pancreatic polypeptide; PYY – peptide YY; NPY – neuropeptide – Y; ARGP – agouti related-peptide; CNS – central nervous system; POMC – proopiomelanocortin; SNS – sympathetic nervous system; GLP-1 – glucagon-like peptide – 1.

Disruptions in many of these circuits instigate the early weight gain after phase one (Holland, 2008). Children with PWS have fasting plasma leptin levels that are significantly higher (Goldstone et al., 2012) and PP levels that are significantly lower than matched controls (Berntson et al., 1993, Goldstone et al., 2012). In contrast, plasma levels of PYY- satiety signalling - are higher in PWS than controls (Haqq et al., 2003a, Haqq et al., 2007) and are negatively correlated with age (Goldstone et al., 2012). GHRT has demonstrated a tendency to lowering PYY levels (Holland, 2008), though investigations suggest that lowering PYY does not determine an alteration in hyperphagia (Goldstone et al., 2005).

Hypogonadism is a core diagnostic criteria in PWS and deficiencies in reproductive hormones (Burman et al., 2001, Festen et al., 2008, Cassidy and Driscoll, 2009) and gonadotropin insufficiency are highly prevalent (Lloret-Linares et al., 2013). In adults, mean serum GLP-1 and cholesterol levels are low but mean serum triglycerides sit within the normal range (Höybye et al., 2002). In the study by Lloret-Linares and co-workers, plasma adiponectin and gastric inhibitory polypeptide (GIP) levels were significantly higher in individuals with PWS. Thyrotropin insufficiency was seen, though the prevalence was not confirmed (Lloret-Linares et al., 2013, Angulo et al., 2015). Central adrenal insufficiency (CAI) has been reported in 60% of children, due to insufficient adrenocorticotropin (ACTH) levels (Van Wijngaarden et al., 2008), though this has not been confirmed (Grugni et al., 2013) and is supported (Beauloye et al., 2015). Leptin also has a role in puberty (Shalitin and Phillip, 2003), which may be why premature adrenarche is common in PWS (Holm et al., 1993, Abreu et al., 2015).

Lastly there is some support for a resistance to type II diabetes. Reductions are noted in brain-derived neurotrophic factor (BDNF) in a small cohort against lean and obese controls (Han et al., 2010) (see all table 1) and in females with PWS, improved central adiposity and glucose metabolism are documented. This is demonstrated even with increases in abdominal subcutaneous adipose tissue as typically subjects with PWS have decreased lean mass (Brambilla et al., 1997, Lloret-Linares et al., 2013) and declining adiponectin levels, over time (Kennedy et al., 2005).

2.3.2 Prader-Willi syndrome animal models

Gene knockout models have been utilized to uncover elements of the genetic loci associated with PWS appetite behaviours (Ding et al., 2008). The human PWS critical region is represented by the mouse chromosome 7C region (Kantor et al., 2006). Deletions of G protein coding genes, *Necdin* (Ndn) and *Magel2* are implicated in disturbances early axonal growth (Lee et al., 2005) and *MKRN3* is implicated in early pubertal development (Abreu et al., 2015). Unfortunately, animal models do not completely replicate the human expression of PWS and some PWS representational animal models have low viability (Herzog, 2012).

Expression of *Magel2* in neurons, is most prominent during the development of the hypothalamus and is implicated in cytoskeletal organization, whereas *Ndn* is implicated in the development of the nervous system. *Magel2* null mice pups have a slowed growth rate, circadian rhythm disruptions and altered food intake and the adults show increased adiposity (Mercer et al., 2013). *Magel2* null mice have demonstrated a loss of depolarization in neuronal responses to leptin within the brain's arcuate nucleus (Mercer et al., 2013), an area associated with appetite. Importantly, these mice experience catch-up weight gain after weaning, which may demonstrate this gene's involvement in the human PWS phase 2, yet *Magel2* targeted mutation models are hypophagic (Mercer and Wevrick, 2009).This unusual discrepancy could be due to reduced locomotor activity or a lower basal metabolic rate. Also, circadian rhythm irregularities have been shown to induce sleep apnoea (Hilaire et al., 2010), which may disrupt the metabolism. *Ndn* null mice embryos show disturbed cortical commissural formation, misrouting of hypothalamic and dorsal thalamus axons and the absence of serotonergic-positive fibres (Lee et al., 2005). Distribution of this gene results in abnormal morphology in medullary serotonin (5-HT) vesicles (Zanella et al., 2008). Treatment strategies may include epigenetic targeting of Ndn, to investigate if re-induction of this gene could establish neuronal migration in the Ndn null mouse model.

Within the PWS genetic loci (Figure 1), there are disruptions of transcriptions which include the SnoRNA genes [small nucleolar RNA (Ribonucleic acid)] located on the introns or

"intragenic region" (a nucleotide sequence within a gene removed by RNA transcription).

SnoRNA *HBII-52* or *SNORD115* and *HBII-85* or *SNORD116* are involved in the alternative splicing of mRNA (Munce et al., 2010). This has important functional consequences, including influencing serotonergic pathways and circadian rhythms.

Though this is obviously not the only gene to show complimentary consideration in regulating homeostasis, there is strong cause to consider the SnoRNA genes as having input in the PWS phenotype. Many of the neurotransmitter serotonin or 5-hydroxytryptamine (5HT) regulatory processes seem to be problematic in PWS as many of the core difficulties involve serotonin regulation within central nervous system (CNS). These include the modulation from the gut to the CNS (O'Mahony et al., 2015), thermogenesis (Schneider and Nadeau, 2015), maladaptive behaviours, sleep and dopamine-related anticipatory appetite (Dimitropoulos et al., 2000, Holland et al., 2003, Lam et al., 2007) and regulation of hypothalamic and pituitary gland hormones (Fulton, 2010).

Whilst the dysfunction of serotonergic signalling is implicated in PWS appetite behaviour, the efficacy of SSRIs in PWS is not clearly established (Kohn et al., 2001) (Tables 3 & 4). In PWS, SSRIs are utilized for OCD maladaptive behaviours such as skin picking (Soni et al., 2007) and are mainly related to therapeutics for anxiety but are not necessarily considered a therapeutic for PWS appetite behaviours. Case studies investigating microdeletions present us with specific questions regarding the role of the genes known to interact with serotonin but unfortunately animal model studies on the 5-HT2c receptor have been thwarted through the vulnerability of the *Snord115* animal models (Kishore and Stamm, 2006, Doe et al., 2009, Morabito et al., 2010)

An important documented outcome of a deleted snoRNA is specifically mentioned due to a deletion of the mouse loci for *HBII-52* or *snoRNA115: mbii-52/Snord115* in the PWS-IC +/- mouse model. Study on this animal has shown that the deleted *Snord115* pre-RNA is specifically related to dysfunctional encoding of the 5-HT2c receptor in the brain (Cavaillé et al., 2000, Wylie et al., 2010).

The 5-HT2c receptor regulates downstream appetite inhibition through POMC activation of alpha-melanocortin signalling via the MC3 and MC4 receptors (Cowley, 2003, Cowley et al.,

2003, Farooqi and O'Rahilly, 2009) (Section 4.4.3.1). The efficacy of these appetite pathways in PWS have not yet been established but clearly it is necessary to determine if the regulatory signals are delayed, weak or thwarted by genetic encoding.

Though micro RNA (miRNA) research is in its infancy, the progression of encoding research has determined that both the PWS loci *Snord116* and *115* have a complex influence during alternative splicing of mRNA coding brain specific 5-HT2c receptors (Schellekens et al., 2015, Canton et al., 1996). The Snord116 is very strongly represented in areas of the brain related to feeding control including the hypothalamic arcuate nucleus (ARC) and the paraventricular nucleus (PVN) (Zhang et al., 2012). *Snord116* mouse models are not as vulnerable as the *Snord115* models though they are consistently smaller than wild type mice (Duker et al., 2010, Qi et al., 2016). This mouse model demonstrates increased hyperphagia after seven weeks (Ding et al., 2008) and up-regulation of neuropeptide Y (NPY) expression levels in the neurons of the hypothalamic arcuate nucleus (ARC). *Snord116* null male mice, have increased insulin sensitivity, as opposed to females, but both have age-related extended bouts of eating, altered metabolism (Ding et al., 2008) and increased respiratory rates relative to intra-abdominal adiposity, leptin and insulin sensitivity (Cnop et al., 2003). Lastly, PWS animal model research may inform our understanding in the regulation of ghrelin related to the environment and comfort food (Schellekens et al., 2012, Steculorum et al., 2015, Zieba et al., 2015).

In conclusion animal models are useful in defining the reciprocity of the genetic loci and for establishing the viability of treatments for single genetic targets. However, they do not represent the full human PWS phenotype for pharmacological evaluation. As yet no animal model is an adequate representational model for the PWS transition to obesity. Human trials are therefore imperative to our understanding for treatment efficacy in PWS hyperphagia.

2.3.3 Prader-Willi syndrome neurobehavioural characteristics

Characterizing the neuronal attributes which determine the disturbed appetite in PWS is difficult due to the many confounding factors. Majority opinion attributes the unremitting hyperphagia to the brain's hypothalamic pathways (Holland et al., 2003, Benelam, 2009)

though mechanistic and causal relationships are not established. In researching appetite and the pathways in humans there is minimal capacity to define these clearly. However, interesting finding are possible utilizing medical imaging techniques.

To discover the neural characteristics of appetite in humans, magnetic resonance imaging (MRI) scans are taken. Function MRI (fMRI), nuclear imaging is a specialized procedure that measures changes in oxygen consumption and blood flow caused by chemical interaction leading to enhanced or inhibited neural activity in the brain (Ogawa et al., 1990). This medical imaging technique and another called positron emission tomography (PET) which also shows the chemical function of the organs and tissues have helped to identify brain regions in PWS that are activated through food-related investigations. FMRI imaging has identified regions activated by appetite-regulating hormones, including leptin, PYY and ghrelin (Batterham et al., 2007, Farooqi and O'Rahilly, 2007, Malik et al., 2008) or through images of food (Pelchat et al., 2004, Bragulat et al., 2010) or even enhanced hunger due to fasting.

Unfortunately, cognitive function may be compromised by psychological factors and early onset morbid obesity (EMO). Though neuroimaging studies control for intellectual disability or OCD, these conditions may still confound the results. For example, changes in routine or restriction instilled from an early age may confuse the subjects. Moreover, the unusual proximity of the food - even as imagery, may incite fear of parental disapproval (even after the parent has agreed to the test). These parameters may result in differing emotional states to obese or lean controls. Similarly, obesity itself may become a limitation as research has shown that obese subjects in the general population have smaller cerebellar volumes than their sibling controls (Miller et al., 2009).

Placing these considerations aside, possible appetite influencing mediators are the neuromodulators mentioned and beyond the hypothalamus; ghrelin's interaction with GH secretagogue receptors (GHSR) or perhaps the structural morphology of the pituitary gland (Jackowski et al., 2011). A reduction in pituitary size has been noted in individual with PWS (Cacciari et al., 1990) as well as those with EMO (Miller et al., 2008). Functional connectivity during fMRI assessment is significantly altered in PWS subjects compared to sibling controls

(Zhang et al., 2013). The intra-cortical organization or gyrification index of complexity is reported to have an association with higher functioning and IQ. A study in children (6-19yrs) determined a lower memory and attention regulation (Lukoshe et al., 2014) in PWS due to reduced frontal, temporal and parietal lobe cortical gyrification within the PWS morphology during structural MRI testing. This aberrant development of cortical folding may have some relationship to the appetite behaviours as detailed in 2.1 and may also be associated with the disrupted early axonal growth due to the deletion of *Ndn* or *Magel2* (Lukoshe et al., 2014, Lee et al., 2005).

Structural differences have also been measured in MRI scans in children with PWS where significant differences in brainstem volume are reported. This may have some bearing on the dysregulation of basal pain perception, sleep and the associated respiratory regulation (Lukoshe et al., 2013).

Utilizing diffusion tensor brain imaging in children and adults in comparison with lean, sibling or EMO controls, complex regional differences are reported (Yamada et al., 2006, Miller et al., 2009, Miller et al., 2007b, Miller et al., 2007a). Differences include smaller cerebellar volumes compared to sibling controls (Miller et al., 2009), neuronal loss or neurodegeneration after the age of five and a reduction in volume in the parietal-occipital lobe (50% of individuals with PWS), ventriculomegaly (100%) and Sylvian fissure polymicrogyria (60%) (Miller et al., 2007a). At a the IPWSO Parents Conference 2013 in

Cambridge Goldstone also reported Miller's findings regarding incomplete insula closure in the majority of subjects with PWS (65%) as opposed to those with EMO. Typically, structural insula closure has some connection to food craving and pain perception, though this cannot be the cause of the early onset obesity in PWS due to there being completed closure in those with EMO.

Reward mechanisms are implicated in the hyperphagia due to enhanced prefrontal cortex activation during food images. Miller and colleagues have reported differences in development of the left frontal white matter and dorsomedial thalamus in PWS during function MRI blood oxygen level dependent (BOLD) tests. They suggest this may have some

bearing on dysfunctional appetite as these areas are connected to the limbic system. However, there is a similarity in response for subjects with PWS to slides showing tools (Miller et al., 2007a).

As reward mechanisms show activation during food image studies there is a rationale for targeting the opioid system in PWS related to the perception of food craving. The prefrontal brain activation documented during food imagery may have a habitual component or could be associated with addiction and reward. It is possible that reward itself is part of the PWS autistic/OCD spectrum, only enlisted because of the slower metabolism and the need for satiety. Studies designed to assess compulsiveness in PWS may further this understanding especially if the appetite is controlled by some means. It would be interesting to assess if the need for reward shifts to a different compulsion other than food. If so, then appetite and OCD could be controlled by dual medications. For example, it may be possible to control obsessive compulsions through medications which calm obsessional behaviours through the opioid system whilst working on mood and appetite with SSRI's alongside a balanced diet.

An alternate opinion to the predominance of reward is that the appetite behaviour in PWS is due to a delayed post prandial satiety response. An enhanced signal is located in the insula, ventromedial prefrontal cortex (VMPFC) and in the nucleus accumbens (NAc) (Shapira et al., 2005) during functional magnetic resonance imaging (fMRI) testing. Interestingly significantly increased firing rates - during fMRI - have also been found in the interoceptive circuitry associated with the insula related to skin picking activity (Klabunde et al., 2015). Though this was only in 10 individuals with PWS this area of the brain may offer some interesting findings in regards to OCD behaviour and interoception or self-perception. Further this area has been associated with delayed satiety in both positron emission tomography (PET) and fMRI appetite studies. Investigations have shown a mean latency for PWS satiation is 24 - 25 minutes, as opposed to 15 minutes for controls (Shapira et al., 2005, Hinton et al., 2006b, Hinton et al., 2006a, Holson et al., 2006, Miller et al., 2007a).

Differences in activity of the orbitofrontal cortex (OFC) (Hinton et al., 2006a, Hinton et al., 2007) are reported in regards to a satiety response against controls. Yet, unexpectedly, similar

fasting states are found in controls when examining neural activation PET responses (Hinton et al., 2006b). Utilizing low and high energy meals (400kcal & 1200kcal meals) - identical in appearance, there was a lack of satiety response especially during the high energy meal. Unusually, half the PWS participants did experience fullness, yet there was a significantly stronger reduction in satiety during the high energy meal (Hinton et al., 2006a). There may be a query here as individuals with PWS rarely eat high caloric content, which to them may be experienced as an unusual or superior taste. Nevertheless, satiety is clearly compromised and hunger is re-established more quickly as the subjective fullness ratings subsided within 2 hours.

An important finding was observed during a fMRI preferred food images study. In individuals with PWS, neural representations of hunger were significantly enhanced during food images viewed postprandially, as opposed to the expected expression of hunger (or interest) pre-meal (Holson et al., 2006). This unusual response was not associated with a dysfunctional signal within the hypothalamus.

Further neurological studies have demonstrated other avenues for appetite treatment. In investigating downstream neural pathways in the PVN and the genetic transcriptional modification of the 5-HT2c receptors' differences in individual polymorphisms have been distinguished through genotyping (Dykens et al., 2011). Tryptophan hydroxylase 2 (TPH2) is a rate-limiting enzyme in the biosynthesis of serotonin. TPH2 polymorphisms have been documented to correlate with a higher IQ and an earlier onset of hyperphagia in PWS. Separately, IQ has also been implicated in the capacity for individuals to distinguish preferences in food (Hinton et al., 2006b).

Lastly in post-mortem studies oxytocin expressing neurons are significantly reduced and vasopressin reduced, yet not significantly, in a small cohort with PWS (Swaab, 1997). It is also suggested that NPY may decrease after the onset of hyperphagia due to lower NPY expression in PWS subjects' post-mortem (Goldstone et al., 2002).

2.4 Treatments in Prader-Willi syndrome

2.4.1 Obesity

There are rising incidences of obesity occurring here in Australia and throughout the world (Kosti and Panagiotakos, 2006). There have been significant increases in the prevalence of obesity in children and adolescents, for example in the USA, 2009-2010, the prevalence was 16.9%. The rise is especially seen in young males (p - 0.04) (Ogden et al., 2012). One might question why obesity is bad? The simple answer is that obesity can cause a risk to health (Kelly et al., 2013). The body's action of storing visceral adiposity or central fat deposits to protect organs create disease when imbalanced by a more sedentary lifestyle. For instance, elevated triglyceride may cause cardiovascular disease, chemicals released may create an insensitivity to insulin which could eventuate in diabetes type II mellitus or central deposits could boost levels of inflammation which cause disease often arthritic in nature (Sherwood, 2015). Though treatment for obesity in the general population is difficult, treatment in PWS is more so, as more often than not treatment involves recommendations for a healthy diet and exercise (McLennan, 2004). In PWS dieting is difficult when caloric restriction is already in place and exercise is difficult due to an awkwardness in gate, muscle tone and often fatigue.

Today's food, in a first world country, is absurdly easy to obtain and over-do. The amount consumed often comes down to choice, culture, economics or the inherited capacity to consume, as in PWS. Unfortunately, choice becomes habitual and an individual's daily diet may not always be healthy. It is possible to create a balanced diet due to the incredible variety of knowledge on obesity and nutrition (Koch, 2012, Murray and Pizzorno, 1998, Chopra and Doiphode, 2002) but unfortunately in PWS recommendations in diet are not necessarily enough to sustain weight between "normal" ranges. An individual with PWS is established to need much support to stay within their ideal body weight range. Therefore, treatment to attenuate the appetite and associated behaviours is imperative.

There are many difficulties warranting individualized treatment, clinicians recommend early diagnosis as best practice (Goldstone et al., 2008b, Goldstone et al., 2008a). Following diagnosis intervention protocols have been recommended to be established as early as possible after confirmed diagnosis.

The most recent PWS Association survey reported 25% of participants (n=471 respondents to this question) started food seeking at 1-2 yrs (PWS Association, 2010). Growth Hormone (GH) is the most researched and definitive intervention within the PWS community. GH is also known as a somatotropin and is peptide hormone given to those with PWS in nightly injections stimulate growth and muscle strength. In PWS it is able to likely prescribed by an endocrinologist but this does not impact on appetite. Further guidelines for treatment intervention and management recommend a multidisciplinary team of clinician, physiotherapist occupational therapy, speech therapist and dietician for the medical conditions in PWS but these also do not impact on the experience of hyperphagia. Medical practitioners may maintain health for sensory impairments, sleep disturbances, scoliosis, psychosis and depression (Chen et al., 2007) and difficulties which occur during puberty, require supplementary treatments such as oestrogen and testosterone (Burman et al., 2001). Yet once again these do not impact on appetite. For this genuine and difficult outcome of PWS treatment involves the parent/carer in meal management, limit setting, external food surveillance and exercise (Chen et al., 2007). Unusually antiobesity agents are not advised in any of these guidelines.

2.4.2 Growth Hormone Replacement Treatment in Prader-Willi syndrome

Growth Hormone Replacement Treatment (GHRT) is the most utilized treatment to date in PWS and many of the clinical study participants within study one of this thesis, were administered this treatment daily. In the PWS population, the occurrence of impaired GH secretion has a negative effect on body composition, stature, maturation, gross and fine motor performance, energy expenditure and respiratory function. GH and gonadotropin deficiencies may also partly account for the atypical fat versus lean mass disproportion (Holland et al., 1995). Though GHRT is generally well tolerated within the PWS community, incidences of

mortality (Van Vliet et al., 2004) have led to a comprehensive evaluation of sleep aptitude before administration. Common sleep disturbances such as sleep apnoea are typically attributed to respiratory difficulties or spiked wave ambiguity diagnosed through an abnormal electrocardiogram (ECG), this alongside questionable adenoidal airway restriction, adenotonsillar hypertrophy (McCandless, 2011) requires medical evaluation culminating in a compulsory sleep-study regimen.

GH has impacted positively on PWS, surprisingly even a reduction in skin picking has been noticed from GHRT (Whitman et al., 2002). Even so within a survey of 546 respondents through the international PWS web site, 346 said skin picking was a concern (IPSWO, conference, 2010). More well acknowledged is the clear distinction that GHRT improves muscle tone, growth and developmental attainment, which by extension boosts self-esteem, social parameters and "quality of life" (QoL). Unfortunately, there is no clear evidence to suggest any suppression of appetite or hyperphagia, through GH intervention (Whitman et al., 2002).

A longitudinal pilot study on those with confirmed diagnosis of PWS, treated with GH presented data on the QoL and psychological well-being of PWS patients [(n=13), mixed gender ranging from 20-33yrs]. This study reported significant differences in regards to both scales of physical and psychological well-being as assessed by the 13 treated patients utilizing the PGWBI, Psychological General Well-Being Index and SF-36, 36-Items Short Form Health Survey - the SF-36 questionnaire is a valid, reliable assessment tool widely used in research on health. The eight concepts of health relating to the QoL were: physical functioning and role limitation in physical activities, social functioning, the intensity of bodily pain, general mental health, psychological distress and well-being - from feelings of nervousness and depression to happiness and calm, role limitations within daily activities, vitality - energy and fatigue and subjective general health; with a total score value for SF36. Nine patients completed the questionnaires over the full period of 24 months and all the PWS patients were obese with a body mass index (BMI) that was unchanged within the time allocated for the trial [(BMI=kg/m^2): 46.3±5.7 (range 47.1 -55.4)]. During the term of GHRT administration, IGF-I levels increased significantly. Though QoL increased, of interest to this

thesis is the fact that the self-control assessment scores varied between the parents and the participant's questionnaires. As noted in all of the complexities within the phenotype of PWS, self-control is regarded as one of the most invalidating features. Within this trial parents found their child's QoL improved but this was in conflict with the perception of the child themselves. Increased QoL - feelings of well-being - seemed to be defined by the parent due to the more coordinated aspects of physical agility, mental speed and flexibility, but not from the statement of self-control. The parents may have seen more effect here because of the confidence levels within their child and therefore an easing of their supervisory position (Bertella et al., 2007). Unfortunately, the assessment of a need for food did not establish as good a QoL for the participants.

For parent/carers of someone with PWS, the constant of having to support the individual is a very relevant indicator of QoL. Though QoL is important this thesis aims to assess the ability for the treatment to raise the level of support. Therefore in study one, the parent examines the indicators of hyperphagia - determined by behaviour, drive and severity of appetite within a validated hyperphagia questionnaire (Dykens, 2007). This informs the researcher of the individual participant's feelings of hunger or satiety more than the presenting feelings about QoL through the parent/carer. The informal journals incorporate research on feelings of QoL

2.4.3 Obesity related pharmacological treatment in Prader-Willi Syndrome

Historically there have been only a limited number of studies on pharmacological treatments for typical PWS appetite behaviours. In the following paragraphs and in tables 3 & 4; defined is the effectiveness of all the pharmacological treatments investigated in PWS including both past and recent trials from large and small cohorts. The published literature review (Griggs et al., 2015a) therefore included single case studies and open label studies. This high impact paper reviewed twenty-two studies of treatments for appetite in PWS, which are summarized in tables 3 & 4 and this section. These are the only studies to date in PWS and they raise many questions regarding future treatments and research avenues.

As mentioned, GHRT does not reduce appetite, so the following is only a brief discussion on GHRT and appetite treatment. The *somatropin* infusion study (Tan et al., 2004); examined the effect of GHRT on food intake in a small cohort of males (n=4) with PWS. Treatment lowered plasma ghrelin and PYY, pre-meal, during the meal and post-prandially, inducing significant hyperglycaemia after 210mins in comparison to the placebo. Even so, these decreased levels of ghrelin and PYY were not experienced as satiety. Somatostatin infusion trials demonstrated similar findings to GHRT. The fity-six week cross-over study (De Waele et al., 2008) on the somatostatin agonist *octreotide* (OCT), (4 x i.m. 30mg OCT acetate, every 4 wks) (n=9 PWS), inhibited the elevated secretion of both acylated ghrelin (known to stimulate hunger) and desacyl ghrelin, with no effect on appetite.

In contrast to GHRT, OCT had no effect on PYY. Similarly, there was no effect on appetite in children with PWS when the subjects were required to stay within the proximity of food for a full hour during two pancreatic polypeptide PP intravenous infusions (110pmol.kg/hr) (Zipf et al., 1990).

Quantitative appetite tests are an accepted measure of individual food consumption in PWS, often compared to obese control subjects. During administration of the opioid antagonist *naloxone* (0.8mg total i.m) x 2, 30 minutes and one minute before ad libitum food intake; delayed satiety was observed in the PWS subjects. The cessation of eating was at different times (one subject did cease eating ten minutes into the trial) (Zipf and Bernston, 1987) yet most demonstrated uncontrolled eating. The obese controls also showed different levels of food consumption, but the individuals with PWS significantly continued eating - some for the full hour. Interestingly, additional testing with different timing did show decreases in the amount of sandwiches eaten during an infusion of bovine PP (Berntson et al., 1993) (see Table 3). Though significant, these increases in fullness were experienced minimally in the female subjects (n=10). However, due to the smaller male group (n=3) this difference may be inconclusive.

In quantitative appetite tests, the subjects are required to stay within the proximity of continuously available food and are allowed to continue to eat. Though controlling for typical

restriction by having parents give verbal permission to the subjects to eat, not many of these tests evaluate the psychological discomfort (or not) of this strong deviation from routine. Clearly when someone is genuinely satiated or full, continuing to consume food is unusual, yet PWS anxiety or OCD may have some bearing on the results compared to obese controls.

Regarding weight, early trials of the β - endorphin antagonist, *naltrexone* -antidipsotropic medication (designed to inhibit alcohol dependency) altered weight, behaviour and skin picking but weight-loss or gain corresponded to restriction or free access to food respectively (Benjamin and Buot-Smith, 1993). Another clinical appetite test documented the effect of treatment with the *benzodiazepine* (BZR) agonist *chlordiazepoxide* (CDP) in 12 individuals with PWS and BMI >30, against matched controls (Fieldstone et al., 1998). BZR is a treatment known to decrease anxiety but it may also increase food intake. This appetite study demonstrated a significantly higher food intake in the PWS group ($p<0.0001$) with a delayed satiety ($p<0.001$). Unusually no relationship between acute CDP administration and overeating was established.

The effect of long term use of *mazindol* – an anorexic agent which binds to neuronal dopamine and norepinephrine uptake sites (0.5 - 1.5mg/day) was investigated in 32 obese patients with a very small sub-cohort of individuals with PWS (n=3) (Inoue, 1995) on a higher dose (1-3 mg/day). Even though the treatment seemed to decrease the meal size and prolong the time between meals, half of the obese subjects left this study due to adverse effects and insufficient effect. *Mazindol* over 6 months, caused body weight and appetite inhibition in 2 of 3 participants with PWS, without adverse effects.

More recently *topiramate,* an antiepileptic drug, has been trialled in open label studies for use in PWS (Smathers et al., 2003, Shapira et al., 2004). The literature into Binge Eating Disorder demonstrates that *topiramate* treatment has a capacity to stabilize mood, create weight-loss and control eating (Marx et al., 2003, McElroy et al., 2004). In younger subjects with PWS (ages 10 -19 yrs), weight and maladaptive behaviours were assessed at baseline and then every three months over the year during *topiramate* treatment. One subject discontinued treatment due to a lack of improvement, while results for the other seven showed increased positive behaviour, reduced hyperphagia and weight improvements. Another study on *topiramate*

treatment in adults with PWS (n=9) (Shapira et al., 2004) showed no change to appetite. Other current trials are the Australian study on oxytocin and a *metformin* trial. The oxytocin nasal spray treatment (Einfeld et al., 2014) has not been successful in arresting any PWS behaviours for older children at this time. This may be due to the delivery method and the blood brain barrier (Tauber et al., 2014) . The open-label study on the antidiabetic drug *metformin* (Miller et al., 2014) has shown some efficacy in a pilot study on adults with PWS and EMO. *Metformin* reduced appetite mainly in the females and especially those with hyperinsulinemia, perhaps by increasing insulin sensitivity. Unfortunately, seven of the males with PWS exhibited aggravated and problematic behaviours of food seeking, moodiness and possible increased seizure activity during administration.

Treatments for OCD typically involve a combination of modalities including CBT, pharmacological SSRI treatment and interventions which target mood, sleep and skin picking (Dykens and Shah, 2003, Soni et al., 2007). There are very few trials on the effect of SSRIs on appetite in PWS, with individual case studies demonstrating inconsistent outcomes. For example, during a fourteen year old boy's psychiatric hospital admission: first *fluvoxamine* (50mg to 200mg/day), then *fluoxetine*, strongly decreased OCD behaviours and skin picking, but increased behaviours of aggression and food seeking, (Kohn et al., 2001). Interestingly, after discontinuation of each treatment the aggressive behaviour disappeared and OCD behaviours increased. Another adolescent, who had experienced limited success when treated with *mazindol* and *fluvoxamine,* found that *risperidone:* an antipsychotic (AP) agent and antagonist of dopamine D2 and serotonin 2A receptors, successfully managed his weight and behaviours, in conjunction with CBT (Araki et al., 2010). Weight-loss due to AP treatment is unusual and not seen in the general population. Due to this effect, an evaluation into AP use in PWS was conducted (Elliott et al., 2015). At this time the suggested reason for the weight-loss is that AP treatment enables a reduction of compulsive appetite behaviours in PWS. AP treatment may align with behavioural therapy.

The use of one medication in conjunction with another may secure a more comprehensive inhibition of appetite for PWS. The SSRI *fenfluramine*, demonstrated promising results in weight and appetite reduction in a double blind cross-over trial (Selikowitz et al., 1990), albeit

with limited documentation of age, dose or confirmed diagnosis of PWS. *Fenfluramine* is a benzenethanamine, related in structure to an amphetamine which promotes the rapid release of serotonin (Buczko et al., 1975) and inhibits the reuptake of serotonin within the hypothalamus (Dykens and Shah, 2003). Unusually, parent observations demonstrated an easing in food preoccupation, but no change to skin picking behaviour. This may be because *fenfluramine* treatment in animal experiments has shown an increased firing rate of the POMC neurons, which interacts with the melanocortin pathway to reduce appetite (Cowley et al., 2003). In 2004, *fenfluramine* was withdrawn from the market due to its association with heart valve abnormalities in some adult patients when used in combination with *phentermine (Griffin and Franklin, 1997),* which limits its usefulness.

Clearly when trying new treatments in PWS, caution needs to be taken in regard to unexpected adverse effects or weight gain. Psychiatric adverse effects were documented during a trial (n=6) on the endocannabinoid CBI receptor antagonist *rimonobant* – which is no longer on the market due to similar effects in obese individuals. Though this CBI receptor antagonist has shown weight-loss and decreased appetite in PWS adults, participants demonstrated side effects of anxiety, disturbed sleep, paranoid ideation and psychotic reactions, with 50% of the treatment group withdrawing from the trial (Motaghedi et al., 2011).

On a more positive note; *exenatide* (a drug treatment for type II diabetes with dose limiting side effects in the general public) was investigated in PWS (Sze et al., 2011). Subcutaneous injection of *exenatide* (10µg), decreased PYY without affecting ghrelin or GLP-1, lowered plasma glucose and demonstrated increased satiety with no undue side effects. Unfortunately, participants did not show a significant decrease in appetite as measured by a visual analogue scale. Similarly, a nineteen year old female with PWS and type II diabetes has had her weight and glycaemic control improved by *exenatide* (Seetho et al., 2011) for up to twelve-months, and eventually her weight plateaued and glycaemic control worsened. Caution is necessary as *exenatide* may delay gastric emptying. Also utilizing subcutaneous injections, a recent treatment is entering stage three trials in PWS. *Beloranib* activity is through methionine aminopeptidase 2 (MetAP2) inhibition. Angiogenesis inhibitors have been used against lymphoma (Han et al., 2000), though in PWS this highly potent MetAP2

has shown efficacy The company that funded the study and the treatment has reported that it was well tolerated and showed evidence of effective use in a cohort (n=17) with PWS (Kim et al.). Unfortunately, the retrospective HQ questionnaires were over a two-week treatment period and caloric intake was also changed for the trial. Further trails are needed. Tables 3 & 4 have a detailed analysis of all the past treatment trials in PWS.

Future treatments to be reported (Miller et al., 2015) are RM-493 which is a potent melanocortin receptor 4 agonist, the unacylated ghrelin analog (AZP-531), designed to target diabetic blood glucose levels and at this time our own work is defining the efficacy of a natural extract in hunger control CFE. Lastly it is suggested that hope for PWS appetite moderation lies in future therapeutic medications like *lorcaserin* where selective agonists of the serotonin 2C receptor may indeed show efficacy in PWS.

Table 1. Treatments in PWS cohort of 1-4

Table . Data extraction, study design, population, treatment and outcome measurements for a cohort of one to four

Reference	Study design	Treatment	Distribution: group A & B, gender, age, weight, height and BMI.	A & B treatment method, dose & period/frequency.	A & B treatment measurement, Statistical Analysis *P* value <0.05 scales & timelines	Results or conclusion *P* values, mean , S.D or S.E	Notes, drop out adverse effects discussion
(Zlotkin et al., 1986)	Randomized double–blind Cross–over design with appetite test.	Naltrexone: *β* - endorphin antagonist	4 subjects with PWS (M) 2 (2, age: 16 ± 2yrs, (W) 80Kg 12kg.	50mg/2 x day, 7 days with two day wash-out Plac.: 50m /day acetaminophen for taste	Data day 3 – 7. Meals x 3 & snacks 2 (with more helpings). Measure (W kg/day & BP. Observe alertness/mood (3 x day). *B* - endorphin & hematology. Student *t* test and repeated measures of variance (Bonferroni).	Plasma *β* - endorphin levels were significantly lower during *Naltrexone* vs. Plac. over repeated measure (*P*=<0.01). There was no change in hematological indices or behavioural markers. All gained weight.	Free access to food may have been a confounder. Tria showed clear individual response.
(Benjamin an BuotSmith, 1993)	Single case stud	Naltrexone: *β* - endorphin antagonist and *fluoxetine*	9 yr old boy with PWS. Oppositional behaviours and severe skin picking	Fluoxetine 60mg/d. *Naltrexone* 50mg/d *(*discontinued and started ov twice).	Hospitalized, 900 Kcal diet and stri behavioural program.	*Naltrexone* altered food seeking behaviour. Skin picking completely healed 9 weeks. No effect on weight. Weight *fluoxetine* loss of 22.7kg over 1.5 yrs (before hospitalization).	Skin picking reoccurred after stopping *Naltrexone* administered at night due to sedation
(Inoue, 1995)	Clinical open label trial with comparison to past mazindol trials	*Mazindol:* anoerxiant	3 subjects with PWS (W) and (H) 1: 87kg, 150cm 2: 75kg, 143cm 3: 87kg, 157cm	PWS dose: 1-3mg/day. Comparison trials: 0.5 -1.5mg/day over 24 wks.	4 weekly - body weight measureme over 24 weeks. (Method not definec	Suppressed appetite in case 1 & 2 of tl 3 cases.	Adverse effects over long term treatment: dry mouth, nausea, constipation and sleep disturbanc
(Itoh et al., 1995)	Clinical open label trial	*Mazindol:* anoerxiant	2 female subjects with PWS (W), (H) and (BMI) 1: 68.3kg , 137.5cm and 36.(kg/m² 2: 87.3kg, 141.4cm and 43.7 kg/m² Note: subject 1 no confirmed genetic diagnosis	One or two oral doses mazindol: 1.0 – 2.0 mg/day over 24 wks. Caloric intake 1,500kcal/day and no exerci program.	Standard Japanese growth charts. (Measurement methods not defined Oral glucose tolerance test GH, PRI and FBS. Observations of food seeking (not defined).	Over 10 - 15 weeks, weight loss subje 1: from 68.3 to 58.3kg and 2: 87.3to 78.5kg. Minimal loss after 15 weeks, eventual gain mostly after treatment end. Food seeking decreased in one. Normal glucose and GH	Subject 2: sever decrease in growth after treatment
(Kohn et al., 2001)	Single case stud	Fluvoxamine & fluoxetine: (SSRI's). Phenothiazine	14 year old boy with PWS, (W) 91kg.	*fluvoxamine* 50 – 200mg/day 2 wks. *fluoxetine* - 2 wks. a phenothiazine (low)	In hospital observations	Both treatments aggravated food – related behaviours. *Fluvoxamine:* 3 kg weight gain. *Phenothiazine*: increased hunger & impaired cognition	Asking/telling & skin picking decreased during treatments.
(Tan et al.,2004)	Infusion study double blind, placebo controlled, Randomized cross-over type matched control	Somatropin: (Growth Hormone)	Gp. A: 4 Male PWS subjects age, (W) and (BMI), 25.6 ± 0.4yrs, 70.2 ± 7.1kg and 31. ± 2.9 kg/m². Gp.B: non PW obese (M), 38.2 ± 3.9yrs, no weight , 34.5 ± 3 kg/m²	Intravenous infusion of somatostatin:250mg/hr for 300min or Plac.: 0.9% saline	Overnight fasting, post-prandially, PWS meal fixed (450kCal) supper. Next day infusion, fasting blood samples at 30min intervals during a libitum cottage cheese sandwiches. Measurement: ghrelin, PYY. Comparison: Gp. B over-night fasting, with breakfast (522kCal). Paired and unpaired student *t* test	Treatment acutely lowered ghrelin & PYY compared to Plac. both before ar during meal. (Similar to controls.) Saline plasma ghrelin fell post prandially by *maximum* of 54.0 ± 4.9 (*P* = 0.002 vs. pre meal), treatment fel 27.9 ± 3.5% (*P* = 0.004 vs. pre meal) Treatment caused significant PWS hyperglycemia after +210mins.	Treatment cause no reduction to food intake. Gp. B overnight fasting, **was** instead of sandwiches

References	Study design	Treatment	Distribution: group A & B, gender, age, weight, height and BMI.	A & B treatment method, dose & period/frequency.	A & B treatment measurement, Measurement, Statistical Analysis p value <0.05, scales & timelines	Results or conclusion p values, mean , S.D or S.E	Notes, drop outs adverse effects & discussion
(Araki et al., 2010)	Single-case study & retrospective (behaviour).	*Risperidone:* antipsychotic, antagonist dopamine D2 & serotonin 2A receptors	11 yr. old boy, with PWS, (W)126kg and (BMI) 61.6 kg/m²	Oral administration 1.5mg/day (max), with cognitive behavioural therapy & restricted to 1600kcal/day	ALT (IU/.I), T-Chol (mg/dl), uric acid (mg/dl), HbA1c (%), insulin (□IU/l), leptin (ng/ml). Retrospective behaviour scores.	Weight reduction to 80.4kg BMI 37.1. Mean figures pre & post treatment: ALT (69, 17,) T-Chol (223, 188), uric acid (7.6, 4.5) HbA1c (5.9, 5.1) insulin (54.6, 7.9) & leptin (117, 11.2). Retrospective decrease: verbal violence & self/other harm.	Stated "Efficacy in inhibiting dopamine and serotonin receptors".
(Seetho et al., 2011)	Single case study	*Exenatide* (Alongside treatment of insulin *aspart* & *glargine*)	19 yr. old female with PWS, and type 2 diabetes, (W) 127.8kg, (BMI) 59 kg/m².	*Exenatide* 5µg sc injection daily x 2, increased to 10□g daily x 2. Basal bolus regimen of insulin *aspart* at meals and *glargine* once daily. (Both reduced)	Measurements of weight, BMI daily insulin units and HbA1c at baseline, 6 months and 12 months.	Improvement at 6 months, yet worsening at 12 months. Weight plateaued: 6 months: (W) 92.5kg and BMI: 42, 12 months: (W0 94.4kg & BMI 43 kg/m². baseline; *glargine* 86-90 units, *aspart* 36 units. 6 and 12 months: *glargine* 50 units and *aspart* 20 units. HbA1c: baseline 11.4%, 6 months 9.1% and 12 months 11.8%	No reported side effects.

Table: ALT= alanine aminotransferase, BMI = body mass index, BP = blood pressure, EE = energy expenditure, FBS = fasting blood sugar, gender: (F) = female, (M) = male, GIP = gastric inhibitory polypeptide, GH = growth hormone, Gp= group, HbA1c = glycosylated haemoglobin, (H) = height, Plac. = placebo, PRL = prolactin, PYY= peptide YY, ± SD = standard deviation, ± SE = standard error, SSRI = selective serotonin reuptake inhibitor, T-Chol = total cholesterol, TFM = total body fat mass, (W) = weight, wks= weeks, yrs = years.

Table 2 Treatments in PWS cohorts of 5 and above

Data extraction, study design, population, treatment and outcome measurements for a cohort of five and above.

Reference (by date)	Study design	Treatment	Distribution: group A & B, gender, age, weight, height & BMI.	A & B treatment method, dose & period/frequency.	A & B treatment measurement, Measurement, Statistical Analysis p value <0.05, scales & timelines	Results or conclusion *p values*, mean, S.D or S.E	Notes, drop outs adverse effects & discussion
(Zipf and Bernston, 1987)	Controlled appetite test.	Treatment: nalaxone opioid antagonist	Gp. A: 10 PWS subjects: gender, age, (W) and (H), (M) 4, (F) 6, 14.9 ± 2yrs, 73.6 ± 10kg, (H) 138 ± 3cm, Gp. B: obese 9 (M) 5, (F) 4, 11.4 ± 2yrs, 83.5 ± 12kg and 151 ± 4.8cm	*nalaxone* (0.8mg i.m.) X 2 & Plac. saline (0.8mg) x 2	Tests x 4: Treatment (30min and 1min before test) x 2 injections, Plac. also x 2, then treatment & again Plac. Appetite test: chicken sandwich quarters (30kcal) – amount consumed, ad libitum, 60 mins. Ten min. time bins, analysis of variance 2 groups x 6 (time bins).	Greater food intake in PWS group (P = 0.015). Decreasing food over time, time block effect (P = 0.046). NS. between *nalaxone* and placebo. No effect on food intake.	No difference in group up to 10 mins. Gp. A: some ate up to 100 sandwiches Gp. B satiety at 15 mins.
(Selikowitz et al., 1990)	Randomized blinded cross-over trial	fenfluramine Placebo: lactose	15 subjects with PWS: age: 5.5- 27yrs (mean 14.2), (M) 7 & (F) 8	5-7yrs (10mg 3/day) 6 wks, 8-15yrs (10mg 3/day) 1 week, then (20mg 3/day), 5 wks, >15yrs (20mg 3/day) 1 week, then 5wks (40mg3/day).	Weight: baseline & end of each treatment. Behaviour observation: first 2 weeks and 2 weeks at end of each treatment. Rating: food related, aggressive and self-directed: scale from 0 = absent – 5 = more than 40 episodes. Wilcoxon signed rank test.	Maximum weight loss was 7% (6kg) (P < 0.02 between 2 time points). Food related (P < 0.05), aggressive behaviours (P < 0.025) and self-directed behaviours (skin picking) no change.	Note: *fenfluramine* has been withdrawn from the market
(Zipf et al., 1990)	Controlled blinded randomized appetite test.	Bovine pancreatic polypeptide	10 obese subjects with PWS, deletion (n=8 to complete).	Intravenous infusions, PP (100pmol.kg $^{-1}$.h^{-1}) x 2, 90 min and Plac. saline , 90 min (Randomized spacing)	Overnight fast then 1 hr. after breakfast (275kcal), 1hr appetite test, chicken/salad sandwich & water ad libitum. Serum at -60, 0 & 60mins: glucose, insulin, C peptide, GIP, PP, glucagon, & cortisol. Repeated measures (15 min bins).	Marked increase in PP concentrations but no effect of PP infusion on other markers compared to Plac. Sandwiches consumed: fasted 63.4 ± 9, PP fed & fasted (51.9 vs. 58.5, P =0.05). NS. effect for drug interaction (P = 0.18).	
(Berntson et al., 1993)	Controlled blinded randomized appetite test.	Bovine pancreatic polypeptide	16 obese subjects with PWS, deletion (n=13 to complete), (M) 3, &, (F) 10	Intravenous infusions, PP (50pmol/kg/h) x 2 = 1 at 0800-0930h & 1 at 16.30-1800h, min and Plac. .saline x 2 (AA,BB) balanced between subjects.	Overnight fast then breakfast (300kcal) at 0830 and 1hr appetite test at 1700-1800, chicken/salad sandwich & water ad libitum. No other food between. Serum immediately prior & 30 mins after conclusion of 90 min infusion. Serum PP levels: repeated measures Plac. vs PP x 4 (15 min bins)	Marked increase in PP concentrations. Plac. Control test sandwiches consumed: 59.8 ± 5.3, PP infusion 52.7 ± 4.7, 12% reduction. 2% in 1st block and 27% reduction in 2nd block. Gender difference (F) -10.8 ± 3.6 sandwiches (M) +4.8 ± 2.1	Note: full results from 11 subjects.

Reference (by date)	**Study design**	**Treatment**	**Distribution: group A & B, gender, age, weight, height & BMI.**	**A & B treatment method, dose & period/frequency.**	**A & B treatment measurement, Measurement, Statistical Analysis p value <0.05, scales & timelines**	**Results or conclusion *p values*, mean, S.D or S.E**	**Notes, drop outs adverse effects & discussion**
(Fieldstone et al., 1998)	Double-blind randomized cross–over appetite test with post-test comparison.	Benzodiazepine receptor argonist, chlordiaepoxide anxiolytic & possible food stimulant	Gender, Age, (W) and (BMI). Gp..A: 12 PWS subjects, (M) 3, 15.7 ± 2.4yrs, 72.9 ± 9.8kg 32.3 ± 6.9 kg/m², (F) 9, 22.3 ± 1.9yrs, 96.6 ± 12.5kg , 44.8 ± 4.8 kg/m² Gp.. B: n=11 obese (M) 6, 12.7 ± 0.9yrs, 101.9 ± 10.8kg, 40.4 ± 3.0 kg/m², (F) 5, 12.4 ± 0.9yrs 89.3 ± 7.1kg (BMI) 35.0 ± 2.7kg/m² and Gp. C: n= 13, adult reference, BMI< 27.5	*Chlordiazepoxid*e HCI (CDP *Librium*) capsules administered orally, one hour before testing. One-day washout between treatment of Plac. 0mg, *Librium* 5 or 20mg/day.	Sandwich quarter appetite test. Serum samples (2 ml) before breakfast (275kcal) and drug administration, then straight after appetite tests (3 x over 5 days). Minute values condensed into 15 min blocks. Group A & B, mixed analysis of variance (ANOVA) x 3 dose x 4 time blocks. Plus, posttest CDP metabolites A & B. Comparison analysis to Gp. C.	PWS subjects ate significantly more food ($P < 0.001$) throughout. 0mg sandwich quarters eaten, PWS Gp. A (50.6 ± 6.8 SE), Gp. B (10.8 ± 2.4) ($P < 0.001$) & Gp.. C (8.8 ± 2.7) ($P < 0.001$). Treatment dose had NS. effect on food intake in Gp. A or B. Posttest CDP serum, showed progressive increase with dose.	Gender was priority for matched controls leading to very different age, weight and height parameters.
(Durst et al., 2001)	Prospective open-label study	*Risperidone*, antipsychotic medication	7 subjects PWS ages: 15yrs & 18-25yrs, (W) 84.3 ± 21.4kg. (one on valproic acid, one pancreatic bypass)	*Risperidone* dose: 0.5mg – 3mg/day, (mean 1.64mg/day) 37 wks.	baseline x 2 visits and post 37 weeks. ROAS, AS, CGIS & weight measurement fortnightly.	Decrease in food seeking with easier communication (observation), ROAS from usual verbal abuse to sometimes, AS baseline 15.1 ± 1.3 and 37 wks: 5.1 ± 1.9 & weight loss to 77.4 ± 15.7	No adverse effects. Pancreatic bypass patient maintained high caloric feeding due to weight loss.
(Smathers et al., 2003)	Open-label study	Topiramate: Antiepileptic or anticonvulsant	8 subjects with PWS, (M) 4, (F) 4 age: 10 -19yrs, (deletion 5, UPD 2 and 1 translocation.)	Dose between 100mg/day – 600mg/day (maintained on "lowest effective, tolerated" dose)	Clinical visit, 8 weeks, 3, 6, 9 months and each year thereafter. Mood and behaviour defined by questionnaires and phone survey. Skin picking by physical observation.	Reported (no figures) positive changes in mood (n=7) and skin picking (n=2), one worsening. Improved compulsive eating (n=3), decreased appetite (n=2). Moderate improvement in compulsive eating (n=2) slight (n=2). All subjects reduced weight gain or had weight loss.	One drop out due to no improvement. Effects of "increased somnolence" (n=3) corrected with dose.
(Shapira et al., 2004)	Open–label trial	Topiramate: Antiepileptic or anticonvulsant	9 subjects with PWS, age: 28 ± 6.7yrs, (M) 4, (F) 5 (BMI) 30.1 ± 4.6 kg/m². (deletion 6, UPD 2 and 1 N/A.) (n=8 completed)	Dose: 25mg, increased weekly by 25mg to 162.5mg ± 23.15 administration at bedtime with concomitant psychotropic meds.	"Screening phase" (1 week) clinical interview, assessment and history. Weekly clinic x 8 weeks. Thereafter 3, 6, 9 months and yearly. Y – Bocs, GDS, COWAT, SCNT, ABC & modified SIB-C assessments (group home operators). Appetite test:1hr free access to food, baseline, wk. 2, 4 & 8. VAS with images. *T* test (slope from zero).	No change to appetite or BMI. VAS appetite increase. No change Y-BOCS. ABC significant changes in behaviour (P= 0.03), self-injury IE. skin picking improvement (p = 0.006). No change in COWAT, lastly SCNT improvement trend (NS.)	One dropout during appetite tests (participants were allowed to finish eating at any time). 900 - 1200kcals/day baseline

Reference (by date)	Study design	Treatment	Distribution: group A & B, gender, age, weight, height & BMI.	A & B treatment method, dose & period/frequency.	A & B treatment measurement, Measurement, Statistical Analysis p value <0.05, scales & timelines	Results or conclusion *p values*, mean, S.D or S.E	Notes, drop outs adverse effects & discussion
(De Waele et al., 2008)	56 week prospective, randomized cross-over trial.	Octreotide: somatostatin infusion	9 PWS subjects age: 14.6yrs (10.8 – 18.9yrs) BMI >85th centile for chronological age. (6 deletion, 3 UPD) Note: one subject >74th centile, included due to hyperphagia.	4i.m. injections of 30mg *octreotide* acetate or saline at 4 week intervals, with a wash out of 24 wks.	Anthropometric measurements, weight, 12hr fast, BMI body composition x-ray, OGTT, HbA1c, IGF-1, ghrelin, PYY, insulin and glucose 30, 60 & 90 mins. on all 10 visits. Food intake 3-day parent food record, Q/A - eating disorder, CBLC, CYBOCS & VAS. Non parametric for two Wilcoxon, pared *t* test or ANOVA for repeated measures.	No change: body weight, food intake or appetite behaviours. In 5 subjects' *octreotide* caused a significant decrease in desacyl ghrelin, 0 (fasting) to 30 min (peak) ($p = 0.043$). No change with placebo. There was significant decrease in 0 (fasting) to 30 min (peak) insulin response ($p = 0.008$) and NS. increase in PYY following glucose intake.	One subject discontinued after first treatment due to acute psychosis
(Motaghedi et al., 2011)	Double blind, randomized, controlled pilot-study terminated early	Rimonobant: endocannabinoid receptor CB1 antagonist	Random sample of 10 subjects with PWS age: 23.73 ± 5.14yrs, (W) 99.58 ± 16.46kg and (BMI) 42.89 ± 8.90 kg/m². Gp. A: Rim. (n=6) and Gp. B: Plac. (n=4)	20mg/day	Measurements: baseline, 90 and 180 days (W), BMI (kg/m²), TFM (g/cm²), Q/A, FRPQ. FBS: ghrelin, leptin & IGF-1 & IGFBP-3 levels. Nonparametric Wilcoxon rank-sum test & time point, signed-rank test	Baseline: BMI (kg/m²). *Rimonobant* 40.7 (31.9-58.7) (n=4). Plac.37.66 (36.6-50.82) (n=4) 180 days: *rimonobant 32.51* (32.50-47.56 (n=3). Plac. 38.81 (35.6-51.31) (n=4). *Rimonobant* and Plac. Fasting ghrelin increased, leptin as per fat mass and *rimonobant* IGF1 levels increased.	Adverse effects: paranoid ideation and psychotic reaction, 50% of subjects on treatment withdrew / study terminated
(Sze et al., 2011)	Single blinded, randomized, cross-over pilot study	*Exenatide* GLP-1 agonist	Gp A: 8 PWS subjects, gender, age, (W) and (BMI): (M) 5, (F) 3, 30.0 ± 2.8yrs, 89 ± 9kg, 37.4 ± 3.4 kg/m². Gp B: 11 matched obese (M) 6, (F) 5, 31.3 ± 2.7yrs, 95.6 ± 2.4kg and 34.4 ± 1.3 kg/m²	Either single sc injection *exenatide*. 10 µg, or normal saline, 15 min before standard breakfast (600kcal). Randomized treatment or placebo 2 wks apart.	Clinical visits two weeks apart. Blood samples, immediately before eating then 15, 30, 45, 60, 90, 120, 180 and 240 min. Body composition dual x-ray absorptiometry, Central abdominal fat and resting EE fasting (-45 to -15mins). Adapted VAS questions and images, rated at -60, 0, 30, 60, 120, 180 and 240 min. Measurements: insulin, PYY total, PYY (3-36), GLP-1 & ghrelin (total). Analysis of covariance or multivariate analysis of variance & Fisher's exact test.	Baseline: PWS ghrelin levels higher than Gp. B. ($p = 0.01$). During meal *exen* sig. increased satiety ($p = 0.003$), NS. effect on hunger. PWS resistance to hunger effect ($p = 0.01$). *Exen* abolished meal response of PYY (total) ($p < 0.0001$) & reduced PYY (3-36) ($p = 0.002$) & GLP-1 ($p = 0.01$), NS. effect on ghrelin ($p = 0.11$). NS. group meal interactions. Pre and meal, *exen* sig. reduced glucose and increased insulin & C-peptide. Plac. postprandial insulin insensitivity in PWS ($p = 0.001$).	Gp. A: no side effects. (questioned high pain threshold & limited vomiting). Gp. B: 80% bloating, nausea or vomiting. May delay gastric emptying. Results: EE, triglycerides, fat oxidation available.

Reference (by date)	Study design	Treatment	Distribution: group A & B, gender, age, weight, height & BMI.	A & B treatment method, dose & period/frequency.	A & B treatment measurement, Measurement, Statistical Analysis p value <0.05, scales & timelines	Results or conclusion *p values*, mean, S.D or S.E	Notes, drop outs adverse effects & discussion
(Miller et al., 2014)	Open-label pilot study	*Metformin* oral antidiabetic	Gp A: PWS subjects gender, age, IQ, (BMI) z score: (M) 3 (F) 11, 11.21 ± 3.81yrs, 84 ± 15.60, 1.70 ± 1.2. Gp B: EMO (M) 5, (F) 5, 11.16 ± 4.35yrs, 90.25 ± 23.76 and 2.59 ± 0.63	Treatment for variable lengths of time (6 mths – 3 yrs)	Standard OGTT, glucose & insulin levels serum 1 & 2 hrs after glucola ingestion. Body fat - x-ray absorptiometry & BMI. HQ for PWS, 11 question, 5-point Likart scale for drive, behaviour and severity (Dykens, 2007) with 3 added questions baseline and after (at least 3 mths) treatment.	OGTT: Responders to metformin had higher 2-hr glucose levels 7.48mmol/L vs 4.235mmol/L (p =0.003) & higher Dropouts:7 of 10 Gp A (M) worsening of behavioural problems after 1-2 days. Gp A PWS (n=13) = 3.08 (p < 0.009), GpB EMO (n=5) 3.76 (p < 0.01). Sig. change in behaviours & food-related anxiety.	Gp A. PWS (M) 7 dropouts due to severe emotional instability and met possibly increased seizure activity.
(Einfeld et al., 2014)	Double-blind randomized controlled trial	Oxytocin nasal spray	Gp 30 subjects. Analysis (n=22) Grp 30 - Age 12-29 mean 18.8 ± SD 4.77 (Del 9, UPD 10, imp. 1).	Dose over 8 wks. Higher dose (n=19) lower (n=11)	(DBC-M (monitoring version) monitors selected items on a daily basis, HQ, (pre-mid-line and post treatment) & informant Y-Bocs, ESS & RMET 45 mins after first dose.	Only change was significant increase in temper outbursts (p =0.023).No reported side effects	
(Kim et al., 2015)	Double-blind randomized controlled trial	*Beloranib* a selective and highly potent MetAP2 inhibitor	17 gp with confirmed PWS Mean 33.5 years, weight 71.8 kg, and BMI -31.4 kg/m2 (completed the study all doses) One subject each in the Beloranib 1.8 mg and placebo groups had type 2 diabetes (no insulin)	SC injection twice weekly *Beloranib* 1.2 or 1.8 mg or matching placebo (1:1:1)	2 wks, lead-in period single-blind, placebo– 4 wk, Double-blind, randomized, treatment period at either dose or placebo. HQ t-test treatment vs placebo one questionnaire for the past 2 weeks.	Improvements (p<0.05) in cardio-metabolic markers occurred with *beloranib* vs. placebo. No significant changes were observed for total cholesterol and triglyceride. Dose dependent decrease of fat mass. HQ food related behaviours (p=0.025 vs. placebo.	Stringent calorie restriction at baseline (~800–1000 cal/day). Minimal measure of HQ.

ABC = Aberrant Behaviour checklist, AS = Aggression Score, BMI = body mass index, CBCL = child behaviour checklist, CGIS = Clinical Global Impression Scale, COWAT = Controlled Oral Word Association Test, CYBOCS = Child Yale-Brown Obsessive Compulsive Scale, EE = energy expenditure, exen. = *exenatide*, FBS = fasting blood sugar, FRPQ = food-related problem questionnaire, GDS = Gordon Diagnostic System, gender: (F) = female, (M) = male, GIP = gastric inhibitory polypeptide, Gp= group, Del = deletion, UPD = Uniparental disomy, Imp =Imprinting error, HbA1c = glycosylated haemoglobin, (H) = height, IGF-1 = insulin-like growth factor – 1, IGFBP-3 = insulin like growth factor binding protein, GLP-1 = Glucagon-like peptide – 1,Sig. significantly, N.S. = not significant, OGTT = oral glucose tolerance test, Plac. = placebo, PP = pancreatic polypeptide, PYY= peptide YY, Q/A = question and answer, ROAS = Retrospective Overt Aggression Scale, SCNT = Semantic Category Naming Test, SIB-C = Self-Injury and Self-Restraint Checklist, ± SD = standard deviation, ± SE = standard error, SSRI = selective serotonin reuptake inhibitor, TFM = total body fat mass, VAS = Visual Analogue Scale, (W) = weight , Y–Bocs = Yale-Brown Obsessive-Compulsive scale checklist, Developmental behaviour checklist =DBC, HQ = Hyperphagia Questionnaire (Dykens et al., 2007), Yale-Brown obsessive Compulsive scale =Y-Bocs, Epworth sleepiness scale = ESS, RMET-(Baron Cohen et al, 2001), wks= weeks, mths= months, yrs = years, Early-onset morbid obesity EMO.

2.5 Caralluma Fimbriata

2.5.1 The Background of Caralluma Fimbriata

Caralluma fimbriata extract is a natural appetite suppressant (Kamalakkannan et al., 2010b), well known in Ayurvedic medicine (Kuriyan et al., 2007). *Caralluma* is planted as a boundary in gardens and is growing wild along roads as a shrub in India, Pakistan, Afghanistan, the Canary Islands, Sri Lanka Arabia and in some parts of Europe. In India's hot and humid climate, the hardiness of this cactus succulent, has seen the plant ingested amongst tribal populations for many centuries. In Andhra Pradesh, Karnataka, and Tamil Nadu of India, it is planted as a roadside shrub and boundary marker in gardens. Almost in confirmation of its availability, this natural medicinal plant flourishes wild in in urban centres and has been used as a vegetable substitute in times of famine (Kuriyan et al., 2007).

The folklore claim of this edible succulent has been its use for centuries in native Indian diets, with claims of appetite suppressing qualities. It is a genus of a flowering succulent within the dogbane family, Apocynaceae, also reported to be part of the family Asclepiadaceae, which as a plant species is noted to be a rich source of pregnane glycosides (Kunert et al., 2008) (Komarnytsky et al., 2013b). The phytochemical ingredients also include saponin glycosides and bitter principles (Figure 4).

Reduction of hunger by a commercially available powdered cactus succulent compound was first claimed through trials on the administration of *Hoodia gordonii* (Figure 5)(MacLean and Luo, 2004c, MacLean and Luo, 2004b, Vermaak et al., 2011), which attributes the anorectic effect of appetite to the steroidal glycosides (Figure 4). This thesis is unable to study the effects of *Hoodia* in Australia, due to difficulties within the registration and patenting of this plant extract. *Hoodia* was traditionally ingested for endurance and appetite inhibition on hunting trips by the Bushmen of the Kalahari Desert in South Africa. This history has created a dispute over ownership, influenced by the San people and Kalahari Desert land rights (Vermaak et al., 2011).

Similarities between CFE and the traditional African cactus succulent *Hoodia gordonii* have been found after repeated chromatographic isolation of individual pregnane glycosides (Figure

5) (Kunert et al., 2008). Eleven novel pregnane glycosides - four comprising of a new pregnane type genin - have been isolated from *Caralluma fimbriata* (Figure 4). The compound steroidal saponin in C*aralluma* resembled the structure of the supposed active compound P57AS3 in *Hoodia gordonii* (Kunert et al., 2008) - which is the patented appetite suppressor in *Hoodia* (Figure 4). *Caralluma fimbriata* & *Hoodia gordonii* are also called slow-growing milkweed succulents. Similar swamp plant milkweed *Asclepias incarnata* has also been boiled and eaten as vegetables throughout eastern and Midwestern America by indigenous groups (Komarnytsky et al., 2013a). The major pregnane glycoside involved in appetite suppression in this milkweed plant is constituent, 12β-cinnamoyl-3,8,12,14β-tetrahydroxypregn-5-en-20-one glycoside (ikemagenin). This constituent has been found to dose dependently increase satiety at two doses (50 and 100 mg/kg/day) in fed and fasted rats (Komarnytsky et al., 2013c).

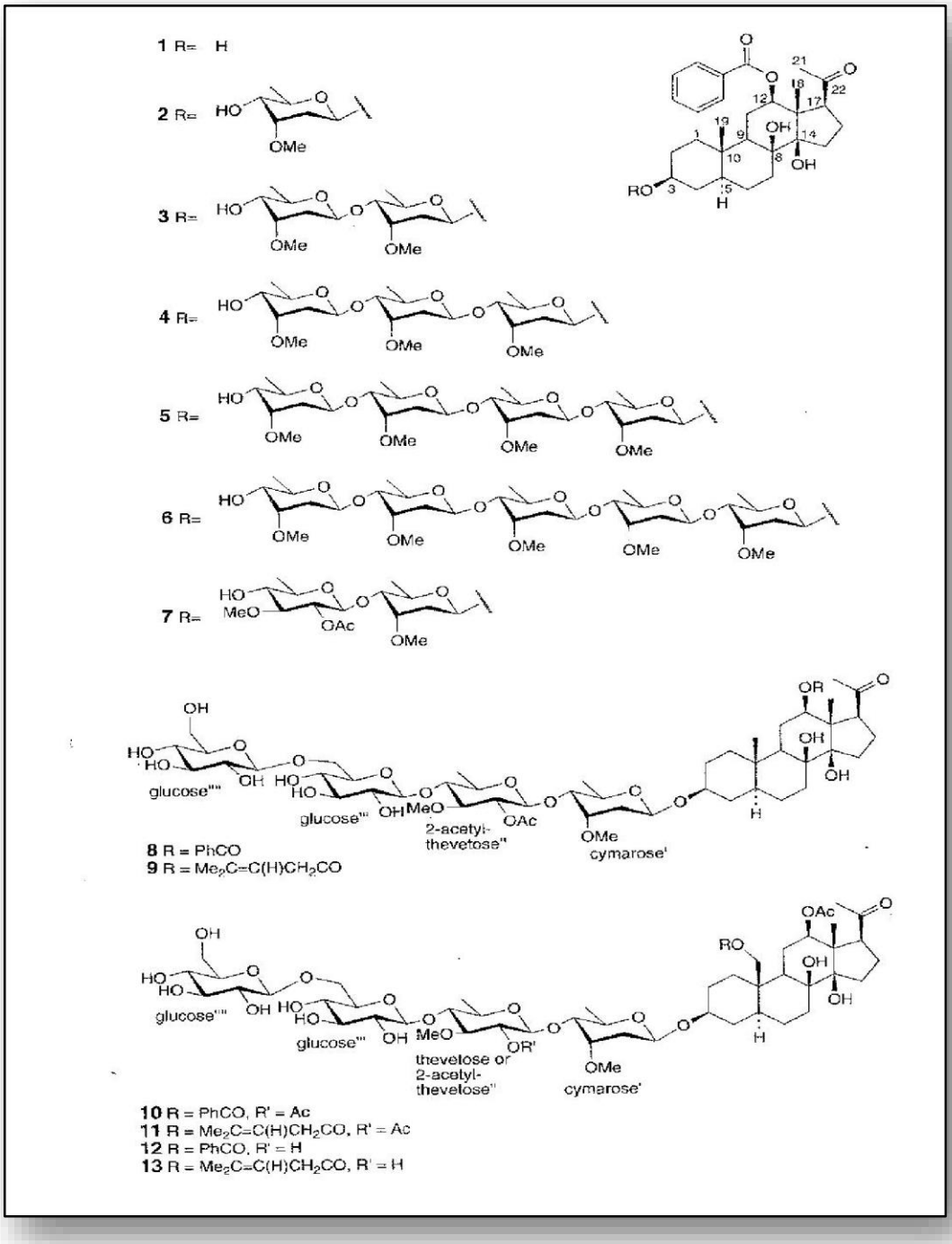

Figure 4 Caralluma Fimbriata Molecular Structure - (ledger next page)

Compound structures of the 13 pregnane glycoside in Caralluma fimbriata-succulent perennial herb. R= radical, (a compound structure such as a hydrocarbon chain or alkaline-like groups β = pressure coefficient, S = entropy, a=Van der Waals coefficient, for the measure of the attraction between particles.1-7 similar axial orientation, 3 and 7 are a similar biocides with a different saccharide portion, 8& 9 similar glucose chain but 9 had absent aromatic H- and C atom resonances replaced by 4-methylpent-3-enoyl group, 10-11 inseparable mixture, as are 12 & 13 all resembling each other.

Compound identification continued:

1. (5a,17S)-12-0-benzoyl-3β,8β,12β,14β-tetrahydroxypregnan-20-one,
2.(5a,17S)-12-0-benzoyl-3β,8β,12β,14β-tetrahydroxypregnan-20-one-3-0-β-cymaropyranoside.,
3.(5a,17S)-12-0-benzoyl-3β,8β,12β,14β-tetrahydroxypregnan-20-one-3-0-β-cymaropyranosyl-(1→4)-β-cymaropyranoside.
4.(5a,17S)-12-0-benzoyl-3β,8β,12β,14β-tetrahydroxypregnan-20-one-3-0-β-cymaropyranosyl-(1→4)-β-cymaropyranosyl-(1→4)-β-cymaropyranoside.
5.(5a,17S)-12-0-benzoyl-3β,8β,12β,14β-tetrahydroxypregnan-20-one-3-0-β-cymaropyranosyl-(1→4)-β-cymaropyranosyl-(1→4)-β-cymaropyranosyl-(1→4)-β-cymaropyranoside.
6.12-0-benzoyl-(5a,17S)-3β,8β,12β,14β-tetrahydroxypregnan-20-one-3-0-β-cymaropyranosyl-(1→4)-β-cymaropyranosyl-(1→4)-β-cymaropyranosyl-(1→4)-β-cymaropyranosyl-(1→4)-β-cymaropyranoside.
7.(5a,17S)-12-0-benzoyl-3β,8β,12β,14β-tetrahydroxpregnan-20-one-3-0-(2-acetyl-β-thevetopyranosyl)-(1→4)-β-cymaropyranoside.
8.(5a,17S)-12-0-benzoyl-3β,8β,12β,14β-tetrahydroxypregnan-20-one-3-0-β-glucopyranosyl-(1→6)-β-glucopyranosyl-(1→4)-(2-acetyl-β-thevetopyranosyl)-(1→4)-β-cymaropyranoside.
9.(5a,17S)-12-0-(4-methylpent-3enoyl)-3β,8β,12β,14β-tetrahydroxpregnan-20-one-3-0-β-glucopyranosyl-(1→6)-β-glucopyranosyl-(1→4)-(2-acetyl-β-thevetopyranosyl)-(1→4)-β-cymaropyranoside.
10.(5a,17S)-12-0-acetyl-19-0-benzoyl-3β,8β,12β,14β,19-pentahydroxypregnan-20-one-3-0-β-glucopyranosyl-(1→6)-β-glucopyranosyl-(1→4)-(2-acetyl-β-thevetopyranosyl)-(1→4)-β-cymaropyranoside.
11.(5a,17S)-12-0-acetyl-3β,8β,12β,14β,19-pentahydroxy-19-0-(4-methylpent-3-enoyl)pregnan-20-one-3-0-β-glucopyranosyl-(1→6)-β-glucopyranosyl-(1→4)-(2-acetyl-β-thevetopyranosyl)-(1→4)-β-cymaropyranoside.
12.(5a,17S)-12-0-acetyl-19-0-benzoyl-3β,8β,12β,14β,19-pentahydroxypregnan-20-one-3-0-β-glucopyranosyl-(1→6)-β-glucopyranosyl-(1→4)-β-thevetopyranosyl)-(1→4)-β-cymaropyranoside.
13.(5a,17S)-12-0-acetyl-3β,8β,12β,14β,19-pentahydroxy-19-0-(4-methylpent-3-enoyl)pregnan-20-one-3-0-β-glucopyranosyl-(1→6)-β-glucopyranosyl-(1→4)-β-thevetopyranosyl-(1→4)-β-cymaropyranoside.

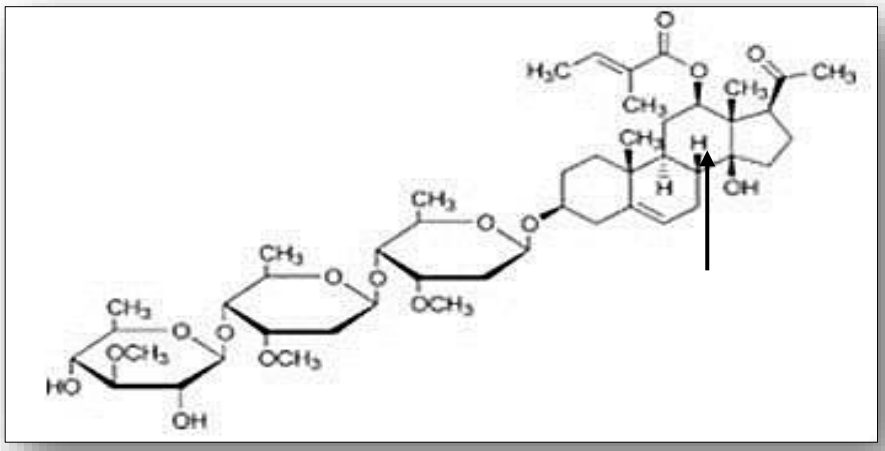

Figure 5 Hoodia Gordonii Active Ingredient

Comparison structure of Hoodia gordonni. Compound structure: 3-0-[β-D-thevetopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranosyl]-12β-0-tigloyloxy-14-hydroxy-14β-pregn-5-en-20-one. Hoodia gordonni is known to be the appetite suppressant patented as P57AS3 (Kunert et al., 2008). The arrow points to the similarity between the structure of the P57AS3 glycoside and Caralluma fimbriata's pregnane glycoside structures.

Of interest to our research into CFE and PWS is a study on *Hoodia gordonii's* active steroidal glycoside P57AS3 which was investigated in a comparative study (Van Heerden et al., 2007). P57AS3 was administered against the pharmacological appetite suppressant *fenfluramine* which has shown some efficacy in PWS (Selikowitz et al., 1990). *Fenfluramine* is a benzenethanamine, related in structure to an amphetamine which promotes the rapid release of serotonin (Buczko et al., 1975) and inhibits the reuptake of serotonin within the hypothalamus.

This treatment had shown encouraging signs in PWS, all be it in minimal studies for appetite behaviour before it was taken off the market (Section 2.4.3 and tables 3 & 4) but more encouraging still was that *Hoodia's* pregnane glycoside appetite suppressing qualities were investigated in a comparative study, in rats against *fenfluramine* (Van Heerden et al., 2007). During the *Hoodia/fenfluramine* trial in animals, there was a significant action of *Hoodia* on food consumption, with an overall decrease in body mass within five days. In comparison *fenfluramine* demonstrated less decrease in food intake and weight gain over the same period. The proposed mechanism for this change was demonstrated in another study on the steroidal glycosides of *Hoodia's* P57AS3 administered by intracerebroventricular (i.c.v.) injection to

rats. In whole cell hypothalamic cultures, P57AS3 inhibited an effect seen when experimental protocols are exploring ouabain binding or Na/K-ATPase activity (MacLean and Luo, 2004b). Ouabain dose-dependently inhibits the uptake of 86-rubidium which is an effect recognized during the mediation of Na/K-ATPase. But when P57AS3 was administered it significantly blocked the inhibition of Ouabain on uptake of 86-rubidium. P57AS3 significantly increased ATP availability to fuel Na/K-ATPase. The reduced food intake of 50–60% lasted approximately 24–48 h, depending on the dose of P57AS3. Liver ATP also showed significant reductions in ATP content (MacLean and Luo, 2004a). Due to this study it is clear that the activity of the pregnane glycosides P57AS3 is hypothetically capable of uptake control and it may therefore be hypothesized that the pregnane glycosides in CFE could show similar action. This interaction is addressed more thoroughly in chapter four in regards to the animal studies and the CNS (Section 4.3.1).

It is believed the appetite attenuation through pregnane glycosides is certainly due to an increased stimulation of the melanocortin appetite pathway (Komarnytsky et al., 2013c) (Section 4.3.1 CNS). Yet though this is obviously a part of the reduction in hunger there are contradictory beliefs and assessments that detail a more complex interaction. For example, (Mammola et al., 2014) it has been reported that CFE increases appetite and fluid intake due to an altered activity of orexigenic neuro peptide Y (NPY) signalling within the hypothalamus. Which is an opposite signal to the melanocortin pathway. This may be the case due to the adjusted adiposity brought on by CFE's capacity to decrease fat cell deposits. Altered leptin signalling may induce a stronger NPY signal (Figure 3 & 10).

The last mechanism suggested within the CFE literature (Kamalakkannan et al., 2010b) is the down regulation or synthesis of Ghrelin from the stomach. This is known to create appetite suppression by modulating neuropeptide Y (NPY) within the hypothalamus. The exploration of Ghrelin levels is of interest for future investigations. Study three investigates CFE's capability of modulating NPY (Section 4 4.3.1).

The hydroethanolic extract of *Caralluma Fimbriata* is commercially available in Australia, titled as a supplement for "Hunger Control". The patented product by Gencor Pacific, is extracted from the aerial parts of the plant, dried and standardized as a powder. The supposed active ingredient are *pregnane glycosides* (no less than 25%) alongside 10% *saponin glycosides* (Odendaal et al., 2013). The patent application for *Caralluma Fimbriata* extract makes the claim that CFE*'s* properties modulate serotonin at the brains synapses

(http://www.freepatentsonline.com/y2005/0202103.html). This thesis demonstrates that CFE does indeed demonstrate SSRI properties.

Whilst there have been studies that identify bioactive pregnane glycosides as compounds with anorectic properties. Questions have been asked of the safety of the pregnane glycosides properties especially when they have increased vacuolization of the adrenal cortex in rats (Komarnytsky et al., 2013a). Various pregnane glycosides structural properties studied in swamp milkweed *Asclepias incartnata* and *Hoodia gordonii* resemble cardiac glycosides seen in *Foxglove* which is administered in cardiac patients during heart failure. However, this is not the role of the pregnane glycosides in *Asclepias incartnata* and *Hoodia gordonii* or indeed in CFE as these pregnane glycosides lack the lactone ring – the stimulating feature - of cardiac glycosides (Komarnytsky et al., 2013a). Figure 4 below shows the pregnane glycoside constituents in CFE.

Comprehensive toxicity assessments have been conducted on CFE (Section 2.4.7) and the safety of this treatment has been assured. In fact in mice a dose of 5000 mg/kg body weight has been reported as safe (Sakore et al., 2012), though questions still arise in regards to complexity of CFE's action. The mechanism of action still confounds. The pregnane glycosides involved in CFE's anorectic action are as follows:

The claim of CFE's expression in the brain has not been validated through research. Questions arise as to the mechanism of action in both weight reduction and documented decreases in appetite. The mechanistic action for appetite regulation is commonly presented through commercial claims. Claims are made that CFE has a significant anti-inflammatory, has anticancer/tumour activity, that it is an antioxidant, an antihyperglycemic, (Priya et al., 2014) and an antinociceptive (Rajendran et al., 2010). Sites suspect that CFE works by different methods but mostly commercial sites determine the activity due to serotonin reuptake inhibition on the hypothalamus.

Therefore, the experimental design of study two and three (Chapters 5 & 6), include experiments to assess the mechanistic action associated with appetite attenuation in animals. Evidence of any SSRI activity may therefore be hypothesized to interact in the process of appetite control in study one's (Chapter 3) children and adolescents with PWS (Griggs et al., 2015b). This will be a target for future study.

There are other proposed mechanisms for appetite control by CFE including the belief that the active ingredient – the pregnane glycosides - are blocking activity through citrate lyase enzyme.

This inhibits the liver's biosynthesis of fatty acids (Dutt et al., 2012). This hypothesis is ventured in a pharmacological review on CFE active ingredient, studied in rats. This proposal was developed after research was conducted on similar compounds. Results from dried fruit rind *Garcinia cambogia* (G. cambogia*)* active ingredient research reported the hydroxycitrate reduces the ATP-citrate lyase which reduces the pool of acetyl-coenzyme A (CoA); which is necessary for the biosynthesis of fatty acids. Hydroxy citric acid is a potent competitive inhibitor of ATP citrate lyase, which is an extramitochondrial enzyme catalyzing the cleavage of citrate to oxaloacetate and acetyl-CoA. Another study conducted on rats (Kamalakkannan et al., 2010b) further described a mechanism proposed for weight-loss. The Kamalakkannan group has demonstrated that the pregnane glycosides inhibit pre-adipocyte cell division during the early phase of adipogenesis. These investigators surmise this to be due to a down-regulation of cyclin-dependent kinase (CDK) or an inhibition of import cyclin D1-CDK/6 complex into the cell nucleus.

2.5.2 Toxicity studies of CFE

For the research on CFE reported herewith, the treatment CFE has come from the original supplier Gencor Pacific Inc. Gencor Pacific gifted the treatment from both India, and the USA. Certificates of Analysis of CFE - with the botanical name of *Caralluma fimbriata* Wall, have been determined from Green Chem, Bangalore India and Gencor Pacific Inc. California USA,

in 2004 and 2008 respectively. Extensive analysis determined the following test values (A= India and B=USA) to show the material complied with the specifications. The powder is A. brown to B. brown to dark brown in appearance. It is specified that it needs to be a minimum of 75% soluble in water, of which it complies A. 98.2% and B.99.1%. Loss on drying, the specification is at a maximum of 10% with both certificates showing 2.6%. Also 98% of the particles need to pass through 20 mesh as derivatives, resinous matter, heavy metals and impurities, i.e. pesticide residue and residual solvents are reported.

Over time the toxicity of this treatment has been rigorously studied. A very thorough safety assessment of CFE in the USA (Odendaal et al., 2013) gave the supplement a no observed effect level (NOEL) finding, in regards to toxicological abnormalities. These toxicity studies support the first evidence of safety mentioned within the Rajendran study where it was reported that animals treated with CFE - up to the dose 2000mg/kg - demonstrated no toxicity.

A trial studying mutagenicity utilizing the extract against sodium azide (2.5µg /plate) and a salmonella strain TA 1535, was found to be able to inhibit mutagenicity. There was also a concentration response in a 1mg of extract / plate, for 500 µg, 250 µg and 100µg, where the inhibition produced was 54, 25, 20 percentages respectively(Sasikala et al., 2015).

In the Odendaal oral study - reported below - the investigators reported no haematological abnormalities from acute treatment of CFE in animals. Also in Bangalore India an acute oral toxicity study reported no change to body weight and no deaths up to 5000mg/kg bw/d. These trials were not designed to investigate appetite or weight gain but the finding that there was no change to food intake was unexpected. Severe weight-loss was mentioned to be seen in a Mumbai Indian trial (Odendaal et al., 2013) at a dose of 270 and 900mg/kg bw/d. CFE was not clearly determined as the cause but what was clear is that during Necropsy it was determined that the four expired animals did not die as a result of toxicity from the CFE treatment.

Four most recent toxicology assessments; published in the *International Journal of Toxicology* (Odendaal et al., 2013), utilize the CFE test article -supplied from Bangalore India, to thoroughly determine the safety of this treatment.

Safety Assessment of the Hydroethanolic CFE:

- A Bacterial Reverse Mutation (Ames Assay), utilizing strains of *Salmonella typhimurium bacteria* for mutagenicity (Test 1)
- An In Vitro Chromosomal Aberration Assay, to assess cytotoxicity (Test 2)
- A six-month Chronic Oral Toxicity Study, in Sprague-Dawley rats, at exceedingly high doses (Test 3)
- And a Developmental Toxicity Study in pregnant females and their embryos in SpragueDawley rats, testing maternal toxicity and fetal development (Test 4).

The methodology of these four studies and their results are as follows: All tests were in accordance with the internationally agreed guidelines of the, Organisation for Economic Cooperation and Development (OECD) for the Testing of Chemicals. For all tests the standardized CFE supplied went through a property determination of the percentage of pregnane glycosides and sapino glycosides, for stability and solubility. The batches content ranged around the 28% for pregnane glycosides and 18% for sapino glycosides. The extract was considered stable at room temperature for approximately five years and >98% soluble in fluid. The dissolved fluid was determined to be homogeneous at 10 -100mg CFE/ml and able to be refrigerated for seven days (Odendaal et al., 2013).

Test 1: Ames test for cytotoxicity, found CFE concentrations to be non-cytotoxic. Guidelines for this testing define ways of determining the response during the testing of different concentrations of CFE in a soluble vehicle. The investigator is looking for a dose relationship increase in nonmutagenic cells. Mutagenicity is assessed utilizing strains of *Salmonella typhimurium* where revertant cell colonies are counted in the presence and absence of metabolic activation through rat liver.

Test 2: the in vitro study also in the presence and absence of rat liver - found CFE to be noncytotoxic. Tests (conducted twice) assessed if there was a clastogenic (structural change in the chromosome) in response to different concentrations, over time (3 or 24hrs) in human blood lymphocytes. A dose dependent cell aberrations was not detected in comparison to negative or positive controls.

Test 3: as well as the fore mentioned NOEL, this six-month chronic animal study determined no structural or functional change in the CNS, no observed physiological dysfunction, and no microscopic abnormalities. In Sprague-Dawley rats (n=40), and an animal satellite group (n=20), treatment at three doses (100, 300 & 1000mg/kg/d) was administered for one-hundred and eighty days, against a control. Clinical examinations were performed before the treatment and weekly alongside recording the amount of food eaten. Immunotoxicity was defined by random blood samples establishing the clinical chemistry, and hematology, plus histopathological examinations and organ/body weights defined the necropsy.

Test 4: the prenatal developmental study was conducted on female Sprague-Dawley rats and showed no maternal toxicity or "treatment related malformations". Mating procedures were performed and CFE was administered from gestation (6 – 19 days) at three doses (250, 500

and 100mg/kg/d) against a control of distilled water. Animals examined twice daily showed no signs of clinical abnormality and after animals were killed, necropsy showed no pathological abnormalities. Pups were delivered by caesarean section and the uterus was weighed and examined for fetal death. Pups were examined for skeletal or soft tissue malformation, of which it was determined were seen in all groups and not as a result of the treatment. All trials determined statistical significance at a *P* value of <0.05.

There has been a study on Albino Wister rats (180-200 g/bw), that determined 5000 mg/kg of CFE to be a safe dose in mice (Sakore et al., 2012). Hence they utilized three doses (250, 500 & 750 mg/kg/d) in rats to investigate the hypolipidemic activity of CFE. Though they determined CFE to have a cholesterol reducing action (Sakore et al., 2012), the Randomization processes of the animals and choice of dose were not clarified. These doses may need further evaluation.

2.5.3 Animal studies of CFE

A study on rats showed that CFE (Kamalakkannan et al., 2010b) exerted a dose dependant reduction in food intake over time up to eight weeks. The trial stimulated pre-adipocyte cell division during adipogenesis in a dose and time-dependent manner (Kamalakkannan et al., 2010b). CFE improved the lipid profile associated with a high fat cafeteria diet and (Arora et al., 2015) significantly reduced the level of leptin in serum. The appetite suppression properties are also hypothesized to occur through a down-regulation of ghrelin synthesis and therefore influence neuropeptide-Y in the hypothalamus (Brunetti et al., 2002). Further lipid profiles were again decreased in another trial on Wister rats. Treatment CFE (100 mg/kg/d) significantly reduced the increasing of body weight during the consuming of a high fat cafeteria diet, in Wister rats, against controls, also significantly reducing blood glucose levels (Ambadasu et al., 2013).

Of interest to the work on CFE and PWS is a paper on the nootropic effect (cognitive/mood enhancement) of CFE in mice (Rajendran et al., 2010). All doses of CFE (250, 500 and 1000mg/kg) induced a significant effect in reducing the escape latency in water maze task performance, suggesting enhancement of working memory and spatial referencing. There was also a significant reduction in anxiety related to the activity sets and the expected alleviation

of food intake was demonstrated through hunger motivated performance experiments. This study provides evidence supporting the use of CFE for hyperphagic OCD behaviour within PWS.

Contrary to this, an unwanted association for treatment in PWS, was described; a reduction in locomotion effect during administration of higher doses. If one was to interpret this as relevant to the humans with PWS a reduction in the ability to ambulate would certainly impede a child or adult with PWS, due to their hypotonic muscle tone. Further slower movement may disrupt the energy homeostasis in PWS as reduced energy expenditure is expected. However, anecdotally and within the clinical study this has not been the case. In Study two within Chapter Five, in the *Snord116* mouse model and WT, the research clearly showed CFE to induce activity; in fact, hyperactivity was demonstrated at the highest dose (Section 5.5.6)

Another unwanted effect of pregnane glycosides is a slowing of gastric emptying reported in rodents (Komarnytsky et al., 2013c). As the introspective experience is dulled in PWS subjects, the ability for CFE to slow gastric emptying warrants careful consideration in regards to supervision for adverse effects. Whilst CFE is being ingested in individuals with PWS considered questions around toileting would be imperative.

2.5.4 Clinical trials investigating the effects of Caralluma fimbriata extract.

The first published experimental study on the effect of CFE between two equally matched groups of healthy adults (n=50, aged 25-60yrs) (Kuriyan et al., 2007) showed no significant effect on weight but a significant effect on waist circumference. The adjustment ($p < 0.001$)

in the treatment group, as compared to the placebo group was through repeated measures overtime, taken at Baseline, day 30 and 60. The trial gave advice in diet and exercise to target 5-10% weight-loss in both groups. Between groups there was minimal effect on weight but interestingly there was a significant effect on waist circumference ($p < 0.01$). This study also found that CFE given at a dose of 1000mg/day, in a twice daily dose of 500mg, (30 minutes before a meal); appeared to suppress appetite over the two-month period. Investigators analysed responses to a 129 food item and physical activity questionnaire. At 60 days "hunger" levels in the experimental group were significantly lowered ($p < 0.001$). An adjustment was not seen in "thoughts of food, feelings of fullness or in the urge to eat" but the change in hunger

was expressed in the intake of the food groups, sugars, cereals, roots, eggs and meat products. This defined a significant reduction in energy 188/kcal day (5.2% carbohydrate, 8% fat and 5.75 protein).

The investigators also examined fasting blood and lipids at the same time points – in the two groups. Both groups had a body mass index greater than 25kg/m² but the results showed no significant change in the biochemical parameters. The adverse effects included gastro-intestinal symptoms including: abdominal bloating, constipation and gastritis. These mild symptoms subsided within a week and were experienced by individuals in both the treatment and placebo groups.

Further to this, two other clinical trials have shown similar findings with evidence of decreases in weight circumference and/or in body weight. The first of these was presented as an abstract at the Annual World Congress on Anti-Aging (Lawrence and Choudhary, 2004) and the second was a trial on CFE, conducted through the School of Biomedical and Health Sciences at Victoria University by Katie Astell, Dr Michael Mathai, Dr. Xiao Su (Astell et al., 2013).

The Lawrence and Choudhary double-blind trial (Lawrence and Choudhary, 2004) (n=26) utilized a stronger dose of 1500mg/day and was unevenly grouped into active (n=19) placebo (n=7). Of those on CFE 88.33% lost weight, compared to almost no weight-loss in the control group (no percentage given). More weight was lost in those obese adult participants with a higher body mass index (BMI) This trial showed limited adverse effects with only a couple male and female – one from each group - complaining of bloating and leaving the trial.

In 2010 a human clinical trial through Victoria University, School of Biomedical and Health Sciences was initiated (Astell et al., 2013).. This was the first trial instigated by the satiety effect of Mia's anecdotal case study of CFE in the single PWS. The results demonstrated similar findings to the earlier studies, though appetite was not shown to be the motivation for weightloss. Overweight and obese participants: adults (n=17) and placebo (n=16), were given an exercise program and nutritional guidelines to follow. As would be expected, this eventuated in weight-loss for both groups but interestingly the treatment group - though showing similar weight-loss, demonstrated a change in waist circumference and an unusual propensity to alter their diet. It is interesting that there was no change in appetite, when the trial demonstrated a significant change in waist circumference. This may be due to the

investigators also giving nutritional advice. The changes made in the participant's regular diet could have camouflaged any change regarding appetite.

Once again when testing for safety and toxicity there were no significant effects in hunger ratings, i.e. fullness or thoughts of food, in a randomized single blind placebo controlled trial, evaluating the safety of CFE (Arora et al., 2015). This trial recruited in overweight and obese adults, CFE (n=47) and placebo (n=42). It is interesting that there was no effect on appetite again in overweight and obese adults.

Trial's especially designed to determine CFE's interaction with appetite in obese or overweight adults seem to expect some adjustment to diet before or during the trial. It may be of interest to continue these adults on their regular diet (without recommended changes or nutritional advice) to discover if the appetite behaviour in the cohort exhibits any natural changes due to CFE and if under these conditions there is appetite suppression. This last trial also begs the question of CFE's commercial claim leading to questions related CFE's constituents when commercially sold to the public.

Lastly there has also been a *Caralluma fimbriata* glycoside activity trial on A-549 cancer cell lines to identify CFE's potential as an anticancer agent {Priya, 2014 #903. Inhibitory activity of 50.32% was defined against increases in cell along increasing concentrations of CFE.

2.6 Key points from literature review

- Prader-Willi syndrome results from a paternal deletion within the critical region 15q11– q13 which determines a genetic disorder with life threatening appetite behaviours.
- The typical expression in PWS follows four individualized "eating phases" which eventually established hyperphagia at the mean age of eight-years (phase three).
- The aetiology of the hyperphagia in PWS is not well understood but is often attributed to central hypothalamic pathways and disturbances within the serotonergic receptors that mediate appetite and satiety signalling (Holland et al., 2003).
- There are many atypical age related hormonal, neural and psychological processes linked to a complex regulation of caloric intake and energy expenditure in PWS. These irregularities are also aligned to compensatory modulated within a system

that is genetically moderated through appetite pathways with unknown neurological capacity.

- Though lowered Ghrelin levels in PWS have not shown appetite alterations, Ghrelin may still modulate within other areas such as the sympathetic nervous system (SNS) (Lambert et al., 2011). Ghrelin levels are raised through anxiety related stress shown through chronic social defeat stress (CSDS) in mice (Lutter et al., 2008) CFE's proposed inhibition of anxiety may alter Ghrelin levels, dropping levels of hyperphagic related OCD behaviour and perhaps appetite.
- To date there have been no successful treatments to attenuate the appetite behaviours in PWS.
- Intervention management guidelines suggest a multidisciplinary team comprising clinician, physiotherapist and occupational and speech therapists (Chen et al., 2007). Food related interventions include both diet consultancy and restriction, balance of energy intake and energy expenditure (Holland et al., 2003).
- *Caralluma's* pregnane glycosides are presumed to work as a selective serotonin reuptake inhibiter within the hypothalamus (figure 15 & 16).
- The use of treatments related to SSRIs have been positively documented in PWS (Selikowitz et al., 1990, Dykens and Shah, 2003).
- *Hoodia gordonii's* steroidal glycosides appetite suppressant qualities has shown evidence of a higher level of "satiety" effect in comparison with *Fenfluramine (Van Heerden et al., 2007)* which has a known efficacy in PWS.
- CFE supplementation in rats improves the lipid profile caused by a high fat cafeteria diet and significantly reduced the level of leptin in serum. CFE evidences the capacity to alter the leptin resistance or de-sensitisation - which is characteristic in obesity (Kamalakkannan et al., 2010b) through changes in adipose proliferation.
- A comprehensive safety assessment has been conducted on CFE. Reports indicate no toxicity up to the dose 2000mg/kg (Rajendran et al., 2010).

2.7 Significance

The PWS Association survey reported that 25% of PWS patients started food-seeking behaviour at 1-2 yrs (USA, 2015). Of course reinforcement of behavioural limits is advised through diet, food rituals and regular exercise. Yet still in 2016 advice mentions (if necessary) environmental modifications or constraints such as locked kitchens, cupboards and refrigerator and external food surveillance (supervision) (Chen et al., 2007) both at home and within the extended community of the individual with PWS.

The key points demonstrate the hypothesized potential action of CFE's regulation through several mechanisms for individuals with PWS. The regulation of appetite, mood and compulsive behaviours in PWS is imperative for QoL in childhood and adolescents, further leading to independence into adulthood in PWS, past the age of eighteen years. The *Snord116* deletion is not the primary causal gene for PWS but it is reported to establish the extremely difficult outcome of hyperphagia from the PWS genetic deletion (Qi et al., 2016). Deletions of SnoRNA's, seen in a vast majority of PWS cases across all the genotypes contribute to the psychopathologies defined by the 5-HT2c receptors regulation of serotonin-mediated behaviour (Kishore and Stamm, 2006).

CFE is hypothesized to interact as an SSRI (Figure 15), which increases activation of this appetite suppressing pathway. For an individual with PWS, any successful contribution to appetite suppression or satiety, is a support for the necessary daily caloric restriction. Any support that adds confidence to the continuation of the daily routine of abstinence, is an intervention worth investigating. Any successful treatment that may be ingested with minimal intrusiveness will be experienced with less hostility or behavioural problems. Any treatment that calms the behavioural problems, mal adaptive behaviours or appetite associated behaviours in PWS, creates support for the individual and the parent/carer of someone with PWS. And lastly any intervention that creates a safer environment around food for the individual with PWS may eventually establish a pathway to an unlocked, free access household a perhaps eventual independence.

CHAPTER THREE

CHAPTER THREE

THE EFFECT OF CARALLUMA FIMBRIATA ON THE APPETITE BEHAVIOUR OF CHILDREN AND ADOLESCENTS WITH PRADER-WILLI SYNDROME

3. Study one

3.1 Abstract

Background

Prader-Willi syndrome (PWS) results from a deletion of the paternal genes in the region of chromosome 15q11-q13. PWS develops hyperphagia over phases, which leads to an excessive ingestion of food when left unmanaged. To date there is inadequate pharmacological treatment or supplementation for modification of the hyperphagia, therefore best practice for management is familial supervision and restriction of diet and environment.

Aim

This thesis aimed to determine if the natural supplement - the Indian cactus succulent - *Caralluma fimbriata* extract (CFE) could attenuate the hyperphagia or the associated appetite behaviours in children and adolescents with PWS.

Material and methods

This thesis conducted a placebo-controlled, double blind, randomized cross-over trial over a ten-week period to investigate the effects of CFE on hunger control, in a cohort of children and adolescents, with confirmed PWS (n=15, mean age 9.27 ± 3.16 yrs**,** body weight 43.98 ± 23.99kg). Australia and New Zealand participants ingested CFE or a placebo of maltodextrin/cabbage leaf over a four-week period, with a two-week wash-out between the cross-over to the other treatment. Weekly comparisons in appetite behaviour, severity and drive

were recorded by parents as scaled time-point measures on a hyperphagia questionnaire validated for PWS.

Results

CFE administration was found to induce a significant accumulative easing of hyperphagia (P = 0.05), with decreases evident in one third of the participants. Furthermore, a significant decrease (P = <0.05) was recorded in the category of behaviour and a decrease in hyperphagia (n=8), (P = 0.009) was observed at the highest dose of CFE 1000mg/d (recommended adult dose). There were no reported adverse effects at any dose.

Conclusion

This thesis demonstrates that an extract of the Indian cactus succulent *Caralluma fimbriata*, improves the appetite behaviour within a cohort of children and adolescents (n=15) with PWS without notable adverse effects. The outcomes of this study have a potential positive impact on PWS management.

3.2 Methodology

3.2.1 Summary of experimental protocol

Study one was a placebo-controlled, double blind, randomized cross-over trial over a 10-week period investigating the effect of CFE on the typical hyperphagic appetite behaviour in children and adolescents with confirmed PWS. Any change was observed and recorded by their parents on a questionnaire validated for PWS (Dykens, 2007), in the timeline defined in figure 6 below, of both the ingestion of CFE and a placebo (PLAC) of *maltodextrin/cabbage-leaf*. Both treatments CFE and PLAC were supplied by Gencorp Pacific International Ltd. The powdered CFE was the commercially-available supplement re-capsuled for our use with a dosage within each capsule of 250mg as the cohort dosage was 250/10kg of body weight, up to and not above the recommended adult dose of 1000mg/d (Kuriyan et al., 2007). Each group A & B ingested the treatment over 4-weeks with a two-week wash-out period in between the two treatment periods. The PWS questionnaires were filled in at baseline, day 9, day 19 and day 28 (end treatment).

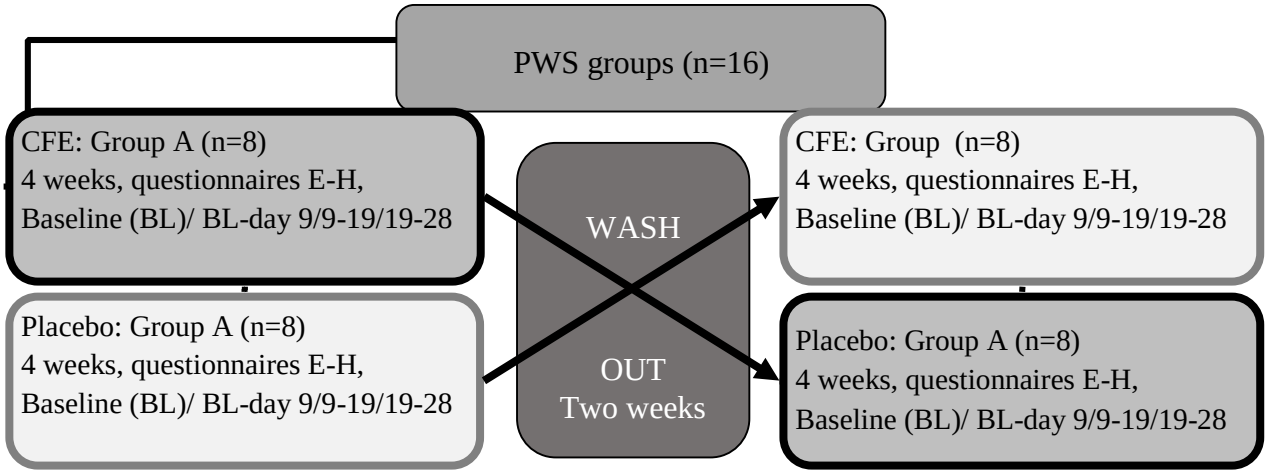

Figure 6 Study One Protocol

Clinical trial design: participant and questionnaire allocation. PWS - Prader-Willi syndrome; CFE - *Caralluma fimbriata* treatment.

3.2.2 Participants, recruitment and inclusion.

3.2.2.1 Registration

The clinical trial was registered through the Australian and New Zealand Clinical Trials Registry (ANZCTR); Registration number: 00336712. Further to this, TGA was submitted a requested notification for CFE supplementation beyond its licensed use.

N.Z. Medsafe confirmed that approval was not obligatory for N.Z. as CFE is "Listed" with the Therapeutic Goods Administration (TGA). Even so they needed to make sure the investigators were not testing the product for commercial medical use in NZ.

3.2.2.2 Ethics consent and inclusion procedures.

The trial was approved by the Victorian University Human Research Ethics Committee on the 1st of July 2011 and an extension was approved on the 30th of August 2013, extending the trial timeline to July 2015. Though *Caralluma* had been licensed by the TGA for use in appetite suppression in 2010, an extra approval was gained related to the ingestion of CFE in children and adolescents. In 2013 New Zealand participation was included due to the rareness of condition and the proximity of finding enough participants within Australia.

Ethical considerations were critical as the subjects were defined within certain categories of vulnerability. There were issues raised when choosing the age of this human cohort. These cohort considerations were:

- The vulnerability of the participants due to endocrine abnormalities when ingesting an intervention that had been minimally researched as a treatment.
- The CFE treatment may have induced adverse effects in children.
- The younger participants may not have been aware enough for consent.
- Dose in a group of this age had never been determined before.

The trial needed to consider supervision. It was important for this investigation that the defined cohort was dependent on parent/carers and the access for observation was rigorous. Also

medications were important for ethical considerations and adverse effects. Most participants were already being administered GH.

It was understood that those recruited would be under medical care for maintenance of their health, therefore GP consent needed to be added without loss of cohort numbers. There needed to be established way of gaining consent in those who didn't have much understanding of their condition without altering the parent's intention of how much they wanted their child to understand. It was expected that in some cases the individuals with PWS would have cognitive impairment or intellectual disability. It was therefore allowed that participants could leave their mark. The consensual protocol was as follows:

- Parental consent was gained for all participants in the trial. PWS participants signed or made their mark as consent.
- Sent "information for participants" outlined details for the parent/carers that were not noted on the participant's, "information for participant with PWS" sheet.

This allowed the parent/carer to decide the amount of information they wanted their child to access related to PWS. Also the parent/carer information sheets outlined all the relevant details about the participant's role in the study before consent.

It is acknowledged that a trial of this nature would naturally address blood serum levels. Of course the relationship between PWS lipid profiles, ghrelin and leptin levels would have been meaningful during CFE administration. Unfortunately, during the ethics process it was determined that the vulnerability of the population was a major consideration. Therefore, when submitting an application through Victoria University Ethics Committee all procedures which created more vulnerability to the participants or extended the concentration on food were restricted. Further the PhD timeline and financial restrictions added a contextual framework to the decisions made in the research plan. Certain criteria were amended from the original plan:

- Including participants within two countries made collecting blood economically problematic.
- Intravenous measurements were thought to be too arduous for the children and adolescents over too many time points.

- The distance to all Australian states and NZ staggered inclusion and lead to measurements being made by clinicians.
- Daily Visual Analogue Scales (VAS) were withdrawn as they meant the participants would have a stronger concentration on food whilst determining how hungry they were.

VAS's became problematic for the parents when assessing questions in the questionnaire. For example, one question in particular asked how much did the participant talk about food or concentrate on food or hunger? To bring the participants concentration to their hunger was contradictory to the questionnaire's use.

3.2.2.3 Recruitment

As PWS is a rare condition and there are only approximately four-hundred recognized cases within Australia, recruitment needed to follow a broad process. Also the study aimed to include a small cohort with enough power for significance. The number settled on after the honours pilot was (n = 16).

Recruitment through the PWS clinic at the Royal Children's Hospital (RCH) was not possible as the RCH would need an ethics submission - which could take up to two years. Therefore, they were contacted out of courtesy and the hospital became a "word of mouth" recruiting point more than an official recruiter. The RCH registry was also unavailable due to a similar consideration. the doctors were emailed the study brochure. The general public was contacted through the Mia Research Foundation (the investigators family foundation) newsletter and the investigator's PWS email lists. An abstract was presented at the Victorian PWS Association AGM and the media was also contacted by Associate Professor Michael Mathai through the Victoria University media department. Newspaper articles and television interviews were deployed in the Herald Sun, on Today Tonight and on a Current Affair

The Prader-Willi Syndrome Associations of Australia, Victoria and New Zealand were informed and a "PWS Protocol for the Consideration of Research", specific to the purpose was sent to the National Association. Recruitment was by distributing ethically-approved brochures on-line and in newsletters within the PWS community. Participants with confirmed PWS; who passed the comprehensive inclusion/exclusion criteria (Appendix A) and gained GP consent were considered for inclusion and were forwarded the medical questionnaire.

Lastly the process of recruitment involved much communication which enabled the participants and parent/carers to ask as many questions as necessary for their full understanding and commitment. Individual recruitment was carefully considered by viewing the answers in the medical questionnaires given to the parent/carers.

Importantly due to the vulnerability of the cohort, their medical conditions and appetite orientated factors considerations of inclusion and exclusion followed strict criteria (Appendix A and B). All the recruited participants (n=16) had diagnostically confirmed PWS and were between the age of five to seventeen years. The lower range limit was to allow for informed consent and the upper range was the maximum age for guardianship. Participants were expected to reside within their typical supervisory environment over the trial period. Accordingly, one participant's data was disqualified as they had transferred parental and environmental parameters.

As indicated by the exclusions (Table 22 Appendix A). Exclusions included people who could possibly experience unexpected adverse side effects due to the severity of an established medical condition (e.g. respiratory disorders, kidney disease) or those on medication. The participating parents needed to be in communication with all involved including the school teachers, school aides and respite workers.

Those who showed an expression of interest (EOI) were sent a plain language statement of the study protocol, mailed to the parent/carer and a similar plain language statement without some of the more severe description of PWS was sent to the proposed participant with PWS. Those applying were able to question the student investigator to ensure they fully understood the requirements of the study. Participants were expected they would gain consent from their general practitioner and were encouraged to talk with appropriate carer/family members. Confidentiality of information was maintained at all times. Once consent was established, the proposed participants filled in a medical questionnaire to determine their suitability and eligibility for the study. The flow chart (Figure 7) identifies the process of recruitment.

The parent/carer (second subject group) was also enrolled. The carers were of course specific to the recruited individuals with PWS. Importantly they spoke English and were able to identify their child's appetite behaviours through their role as supervisor. This needed to be within the home environment as the number one carer. They also needed to be in consultation with any

outside carers. Outside representatives included respite workers, teachers, aides and special school facilitators - anyone placed in a position of direct "one on one" supervision. If the carer was unable to meet these criteria, then the participant with PWS was not included (please see inclusion criteria Table 21, Appendix A /b).

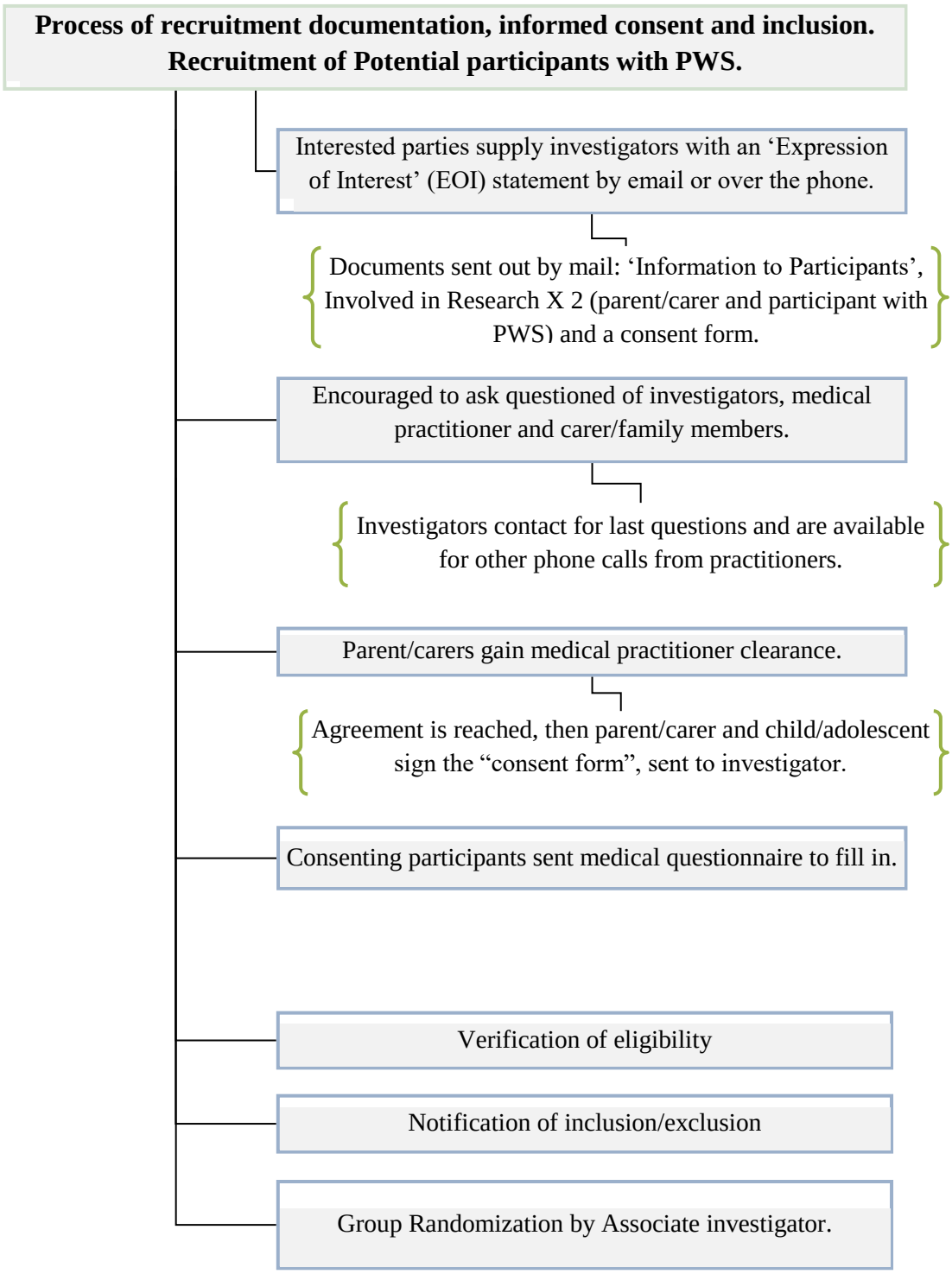

Figure 7. Recruitment Flow Chart

3.2.2.4 Inclusion criteria and exclusion criteria

The inclusion criteria for the participants with PWS may be seen in Table 21, Appendix A. They were defined as: those with a confirmed diagnosis of PWS, ages 5-17yrs and exhibiting many of the phenotypical PWS parameters (Burman et al., 2001). The clinical inclusion/exclusion questionnaires allowed inclusion for those with phenotypical parameters, i.e. sensory impairment or developmental delay and many of the core criteria for PWS. On the other hand, the exclusion criteria (Table 22, Appendix B), outlined comprehensive list: firstly, omitting those without a confirmed diagnosis of PWS and those who did not speak English. Next exclusions were any participants who could possibly experience unexpected adverse side effects due to the severity of an established medical condition (e.g. respiratory disorders, kidney disease, and frequent obstructive sleep apnea).

The medication exclusions were consistent with ethical considerations related to the vulnerability of the population. This exclusion was to minimise any random effects and therefore variables. As GHRT is unavoidable within the PWS population, the medication exclusions did not include GH. Therefore, the medication exclusions were anyone ingesting or being administered a medication other than GH and those individuals on GH. This was however defined within a parameter that made GH would not alter the outcome. Regarding GH, it was decided that the exclusion criteria would also involve anyone who had recently started GH (within two months of their start date for the study). This was due to the inability for parents to recognize the difference between changes induced by GH as opposed to CFE or placebo ingestion.

Regarding the second subject group inclusion, the parents were included if they were able to speak English and classified as a full time parent/carer. The recommended practice - in managing the condition of PWS - suggests a coordinated program through a multidisciplinary team. Accordingly, the participating parents needed to be in communication with all involved including the school teachers, school aides and respite workers.

3.2.3 Experimental protocol

3.2.3.1 Randomization

Randomization was determined by the Associate investigator who had no contact with the participants. Participants, carers and the investigator were blinded to the treatment allocation. As both genders in PWS experience similar difficulties with food restriction, the two groups were randomly separated (n -16, group A= 8, B =8) with mixed female and male participants. The recruited participants were randomly coded and placed into mixed gender groups. The consenting participant's parent/carers were allocated into identical groups (with their child). Here they were directed by the investigator in a blinded protocol related to the participant's treatment administration. The parent/carers were directed on their child's specific allocation (dose for weight) and on how many powdered capsules (CFE/placebo) they needed to place within the breakfast juice daily. Each participant was their own control within the eight weeks of treatment. Randomization continued into the administration as all the results were also coded on questionnaires.

3.2.3.2 Clinical consultation

The first treatment consultations were originally conducted through the Victoria University Teaching (Nutritional Therapy) Clinic, Building 2, St Albans Campus, Melbourne, Vic. Australia. Due to the need for participants to be interstate and overseas these consultations changed very early - after the first three consultations – to include general practitioner (GP) consultation. Therefore, participants were offered the opportunity to define their first anthropometric measurements through a GP clinical consultation. Most of the time these measurements were taken at the time of GP consent. It was also decided that midline and post intervention consultations would be conducted by phone and email. The baseline consultancy weight was utilized to confirm the participants' weight for dosage and the height was mainly utilized to clarify the body type. To minimize the level of discomfort for the children/adolescents, waist or hip circumference measurements were not taken. Weight-loss was not a prerogative for analysis as the trial was trying to minimize the variables of behaviour. None of the anthropometric measures were to be used as statistical measurements.

After inclusion - at baseline - all participants were sent their non-labelled treatment, a capsule calendar - specific to their start date, an adverse effects protocol, eight hyperphagia questionnaires (HQ A –H) (Appendix C) and a supervisor journal. Further at midline participants were sent their second non-labelled treatment without knowing which treatment CFE/PLAC they had received in the first or second instance. All parent/carers were given unbiased instruction on how to document any information given by other carers and on how to identify any changes to behaviour, diet or the amount eaten. The parent/carers were thoroughly instructed on the parameters of the study and on how and when to fill in the hyperphagia questionnaires which they were to do at home within the timeline of their capsule calendar. Hyperphagia questionnaires A (HQA) was filled in at baseline, or within the first consultation. Interstate parent/carers forwarded theirs by mail. There were no instructions given by the investigator (other than the treatment protocol) related to changing the participants' routine or behaviour.

Measurements of parent/carers observations were from their child's daily interactions of hunger and behaviour. These observations included results from discussion with the other parent and other supervisors (grandparents, respite workers or aides) observations, all outlining the severity, behaviour and drive related to food preoccupation. Any changes in appetite behaviour, severity and drive were defined by a numeric representation within the questionnaire as a measurement parameter from a Likert Scale (1 – 5) in severity.

3.2.4 Treatment protocol

3.2.4.1 Supplements and Placebo

Both CFE treatment and the placebo were supplied by Gencor Pacific International Ltd. (Hong Kong). The constituents of *Caralluma fimbriata* (the supplement) were extracted from the aerial parts of the plant with an alcohol solution, granulated, ground and then dried. The powdered extract was then enclosed in 250mg unlocked cylindrical capsules for storage at room temperature. The placebo of *maltodextrin* (glucose polymers) and *cabbage leaf* – to match the

CFE's colour and the bitter taste - were also enclosed in coded 250mg capsules (200mg *maltodextrin* and 50mg *cabbage leaf powder*). The placebo of *maltodextrin* (glucose polymers)

and *cabbage leaf* was added for colour and bitterness was also powdered and enclosed in 250mg capsules.

The capsules were able to be broken open. To prevent choking each participant's coded full dose was dissolved by the parents in a tropical juice for breakfast, while remaining blinded to the actual treatment. Though it is not recommended to give children with PWS, juice, this protocol was important because of our cohort's age, choking hazards and the bitter taste of CFE.

3.2.4.2 Supplement Dosage

After two experimental PWS groups were coded (A & B), and the participant's initial anthropometric measurements - identified for dosage - were checked against medical questionnaires, then these measurements were utilized to create correct dosage. As there were no clinical studies on the effects of CFE, within this population or for that matter in children and adolescents, the supplement dosage was carefully considered due to the anecdotal evidence (appendix 1) and clinical trial information. The optimal recommended adult dose has been found to be 1000mg/d in humans (Kuriyan et al., 2007). CFE has been administered at a higher dose of 1500mg/d (Lawrence and Choudhary, 2004) in humans and has been quantified as nontoxic in animals up to a dose of 2000mg (Rajendran et al., 2010).

As there were no clinical trials in PWS, or for that matter in children, the supplement dosage was carefully considered, the dose was conservatively capped the intervention at the 1000mg/d as this was the recommended adult dose on all commercial packaging and was also the capped dose for subsequent trials in humans (Kuriyan et al., 2007, Astell et al., 2013). For the PWS cohort the lowest dose was delineated as 250 mg at 10 kilogram of body weight and raised 250mg each 10kg above this, up to and not exceeding the recommended adult dose (1000mg/d = 250mg x 4). Therefor for a child of 10kg - 19.99kg the dose was 250mg per day; 20kg - 29.99kg - 500mg per day; 30kg - 39.99kg - 750mg per day and 40kg and above (1000mg/d). The daily dose was ingested first thing in the morning as a single dose.

The placebo's composition approximated the intervention in terms of colour and bitterness. The colour was needed as the participants were opening their capsules to mix them with juice before ingesting them. This placebo was not active in satiety. The specified dose of either CFE or the placebo was assigned to coded containers by the associate investigator. The investigating student communicating with the participants was handed the coded treatments for first four-week period. All participants were sent the treatment, instructions, and individualized capsule

calendar, a mixing cup and an adverse effects protocol by express post. At midline after washout the second cross-over intervention/placebo followed the same process of distribution.

Compliance to the program was assessed weekly by phone or email. Parent/carers were asked to note their child's intake of capsules on the calendars provided. Drop-outs, adverse effects and compliance was recorded and the journal entries were voluntary.

3.2.4.3 Protocol for supply to subject groups

Due to interactions through customs it was requested that a custom assurances was created. This was necessary given the treatments compounds were unusually being sent one at a time overseas and that the delivery needed to be blinded as to what was within. Therefore, documents were sent within the mail to cover any concerns within customs about both treatment and placebo. Customs was concerned with the Medicines Act of 1981 which notes the meaning of "new medicine" as: Any medicine that has *not* been generally available in New Zealand

3.2.4.4 Treatment adverse events protocol

To prevent any anti-nociceptive effect, those with a strong recorded history of reduced vomiting were excluded. Other trials of CFE in non-PWS adults reported occasional moderate upsets which subsided after a week (Kuriyan et al., 2007). Therefore, the adverse effects protocol and weekly communications advised parents to be vigilant in noticing any side effects or gastrointestinal stomach upsets. Importantly individuals with PWS may have a reduced sense of pain(Cassidy and McCandless, 2005) therefore anything regarding a distention of the stomach or an adverse effect were to be reported to the investigator. As those with PWS may have viscous saliva (Saeves et al., 2012) and differences in thirst, drinking could be a problem so parent/carers were advised to monitor their child's fluid intake.

The adverse effects protocol presented to the parent/carers advised a procedure in case of severe adverse events. The protocol gave clear guidelines to stay vigilant in observing of any or severe gastrointestinal stomach upsets or allergic reactions. If these occurred parents/carers were instructed to cease supplementation and to attend a GP clinic at the expense of the responsible investigators. The investigator was attentive to any phone calls from the parent/carers and

instructions informed the parent/carers that they could cease the trial at any time. If the circumstances were adverse or severe they and their child would be removed from the trial.

The adverse effects protocol to parent/carers and participants, stated that withdrawal from *Caralluma fimbriata* would not cause any adverse physical effects (Kuriyan et al., 2007) and Lastly in the event of a participant feeling disappointed or anxious during or after the trial, the cohort were offered psychological counselling post trial. The counselling offered was with senior clinician within the Social Sciences & Psychology group at Victoria University Melbourne.

3.2.5 Measurement

3.2.5.1 Anthropometric measurements.

As reported the initial baseline consultation anthropometric measurements were weight and height through a clinical GP consultation. The second measurement for dose at midline, was a comparison taken at home by the parent/carer (all participants Melbourne, interstate and overseas in NZ) to check the weight for dosage was still within the same 10kg range.

In regards to anthropometric measures, weight was determined by placing the scales (checked for a reading of zero) on a hard horizontal floor surface. The participants were asked to remove their shoes and heavy outer garments. Weight was recorded in kilograms. The weight supplied by a clinical consultation at Vic University or by an outpatient G.P. was checked against the medical questionnaires sent by each parent/carer earlier, to ensure the correct dosage had been allocated.

Height was measured using a stadiometer whilst the participants stood with their heels close to the wall and their calves, buttocks, backs and head touching the wall's surface. Participants were asked to stand with their feet together looking straight ahead at a 90° angle of their chin and their height was recorded to the nearest millimetre, vertically from the base of the floor, along the wall surface, to the top of their heads. Physician documents were used in the case of interstate participants.

To minimize the level of discomfort for the children/adolescents and to delete the variable of distant measurements no waist or hip circumference measurements or body mass index was

taken. None of the anthropometric measures were utilized as statistical measurements as this study was not investigating weight-loss.

3.2.5.2 Hyperphagia questionnaires

The life-threatening and often idiosyncratic appetite behaviours in PWS are determined by common expected markers which may range from repeatedly asking for food, to obtaining food when not allowed. These are well recognized by the parents due to a history of constant attention and supervision. As supervision is mandatory in PWS and all participants were tested for full supervision access, it was possible to test the hypothesis by utilizing a thirteen question hyperphagia questionnaire (HQ) validated PWS (Dykens, 2007) (Appendix C). In this questionnaire known PWS behaviours were identified by the range of measures within the validated questionnaire in a more generalized fashion. For e.g. a child may ask the time often and the parent may know what they are really asking which could be when is dinner? This would come under the category of repeated questioning, or concentrating on food depending on the parent. As the baseline was defined by this same parent/carer, their perception of each question would stay the same throughout.

Evidence of any change in satiety or behaviour was marked within a multiple choice range with a five step gradient from 1 (low – not a problem) to 5 (high - frequently a problem). Measurements were taken at baseline within the thirteen-point questionnaire (week 1 - HQA), during the second and third week (HQB & C), and the day after the end of treatment (week 4 - HQD) as per Table 5. After a two week wash-out this process was repeated during the crossover treatment/placebo (HQE baseline – HQH post-treatment). Data was collected from the thirteen questions, with eleven questions specifically designed to identify changes of appetite over the trial, grouped into severity, drive and behaviour. The other two questions were: the age the hyperphagia was detected (HQ12) and the variability over the trial (HQ13) (Dykens, 2007). No routine was changed during the trial period, i.e. change of school. The placement of the questionnaire allocation was defined below.

Table 5 Questionnaire Allocation

Caralluma fimbriata extract in Prader-Willi syndrome: double blind, randomized, cross-over design, treatment protocol and hyperphagia questionnaire allocation/placement.

Timeline	Group Allocation and Questionnaire Placement
Randomization	Pre-treatment Consultation / Check weight for dosage
Day before treatment	HQA
Day 1	PWS group A (n=8) CFE / PWS Group B (n=8) PLAC
Day 9	HQB
Day 18	HQC
Day 28	HQD 4 weeks
Day 29 - 43	Wash-out (2 weeks)
Day 40	Check midline weight
Day 42	Before second treatment/placebo: HQE
Day 43	PWS group B (n=8) CFE / PWS Group A (n=8) PLAC
Day 52	HQF
Day 61	HQG
Day 72	HQH
Day 73	Post-treatment Consultation

Table 5. Time-line of treatments and hyperphagia questionnaires (HQ). Eight HQ's from A-H. PWS – Prader-Willi syndrome, group, n = number of subjects, CFE – *Caralluma fimbriata* extract, PLAC – placebo of maltodextrin/cabbage leaf.

The parents/carers were to supervise their child's behaviour within each single study week, eg. Between questionnaire A & B (baseline to the beginning if week two) and were asked to answer the questions related to that weeks' timeline. The times were specifically individualized and delineated on the capsule calendar to create reliability. All parents/carers had a complete understanding of their role and were able to answer the questions easily. They also understood they needed to transfer observations from the other parent and from the other parent at home or outside supervisors/carers.

The parametric delineation of the Likert scaling (1-5) on the hyperphagia questionnaire was quantified through the single parent/carer's opinion only. Appetite or satiation was tested by the HQ through generalized PWS behaviours. The data collected by the questionnaire identify increases or decreases in the scale from each baseline, using a well-known range of PWS related behaviours. As is represented by Dykens (Dykens, 2007), the defined questions were also organized into sub-groups of severity, drive and behaviour.

It was challenging that the validated HQ for PWS was designed for a different time line trajectory other than a ten-week trial. Parameters of the questionnaires were designed to reflect change over the long term, therefore it was mentioned to all parents/carers that 'once a year' would represent rarely and "monthly", in the context of one week meant occasionally. As the eight questionnaires were repeatedly answered by the same parent/carer the parent's perception of these meanings would stay relative and constant.

3.2.5.3 Journals

The supervisor journals were mainly used by the parent/carers to record notes on hyperphagia behaviours. Due to changes in care in other environments, for example at school, information from other carers were able to be noted within the journal. The diets consumed were not written down, however, changes to the amount of food or diet were important to the outcome. As those with PWS are on clearly defined restricted diets, it was decided that no nutritional advice would be given; this allowed the diets to remain the same throughout the treatment. Parent/carers noted any tendency towards change due to the treatment and were asked to journal any natural occurring change to the diet regimen. The journals were also used by the parent/carers to record notes on hyperphagia behaviours given to them by other supervisors. They recorded any events within the visual and auditory supervision in all environments; except within the parameters of natural toileting. As the journals were voluntary it was not expected that parent/carers would remain vigilant with writing in the journals. Any information was to be utilized by the investigator as a narrative synthesis.

3.2.5.4 Statistical analysis

Statistical analyses were performed using the IBM SPSS version 20.0 for Windows, and Microsoft Office Excel 2010. Statistical significance was defined at 95% confidence intervals. All data were normally distributed as determined by Shapiro-Wilk's. The data analysis from the HQ factors were defined by operational definitions ranging from low to high (1 -5) as in Section 3.2.5.2.

The questions were grouped into factors of behaviour, drive and severity as per Dykens 2007 (Dykens, 2007). There were thirteen items included within the analysis though two did not load as a factor. The first of these was a question without variation, the age of onset for the

hyperphagia (HQ question 12) which has been correlated in the participant results section and the second was the variability in behaviours from week to week (HQ question 13). Both questions were analysed for any interactions with the results.

Changes to appetite behaviour were evaluated using independent paired, *t*-tests and ANOVA for repeated measures. Eleven of the questions were loaded for parametric delineation. HQ12 & HQ13 were not within the factor analyses. Time-point comparisons of variability from baseline to week four - of each treatment cycle - were analysed and the results were expressed as "accumulative" in mean ± standard deviation (SD), with $P = \leq 0.05$ considered significant.

The questionnaire allowed for a broad overall range in accumulative total score over the eleven questions. Therefore, these eleven questions assigned for hyperphagia measurement were capable of a total score of 55 = 100% hyperphagia (11 x 5), 5 being the highest score possible per question). Further on each baseline questionnaire (HQA & HQE) there was also the capacity for participants to experience no change with 1 in the Likert scale being the lowest score (1 x

11) 1 being the lowest possible score. Subsequently parents who rated their child's hyperphagia at the lowest score at baseline had nowhere to go if treatment did alter their child's appetite. Therefore, the statistical evaluation also ranked the participants by a percentage for their capacity to record a decrease. Baselines were statistically checked for significant difference which reinforced the reliability of the individual participant's accumulative scores against their cross-over treatments. Individual accumulative scores were also analysed to help establish if there were any relationships in regulation due to dose, genotype, weight or early hyperphagic drive.

To determine if there were any significant interactions between the parameters CFE/dose or the participant's genotype, body weight, age of onset or level of hyperphagic drive the thirteen questions were further analysed for future study directions, for example: to increase the dose or change the weight guidelines. Observations of this nature are noted within the discussion of study one, along with the narrative synthesis of the voluntary journals.

3.2.5.5 Administration

Delineation of responsibilities for investigators and recruitment strategies were documented within the ethics protocol and subject placement and retention has been retained by the responsible investigator. Raw data collection, data extraction, statistical analysis and budgets

were clearly documented by the student investigator. In accordance with the code of conduct for research, all data will be retained securely within the Victoria University St Albans campus, Melbourne, for at least 15 years post the pilot study publication date.

3.3 Results

3.3.1 Participant subject group allocation

Though thirty-two subjects were recruited, the demographic characteristics and strict exclusion criteria led to the exclusion of sixteen prospective participants from these enquiries. The reasons for exclusion were:

- Age
- Inability to give consent
- Medications taken - other than vitamins
- Impaired respiration due to sleep apnoea

Eventually the participants and their parent/carer - who met the inclusion criteria (n=16) - were randomized into groups by an external associate supervisor (Griggs et al., 2015b). This supervisor had no knowledge of any of the cohort's identities or parameters.

3.3.2 Prader-Willi subject group results

The final cohort for evaluation had sixteen participants though one was dropped out of the results leaving the final cohort (n=15). The single drop-out was due to change in parental and environmental supervision. This led to a different person's information being the sole supervisory information over a holiday camping period. It was difficult therefore to determine that the results were without variation of opinion between the two treatments.

The results of the 15 participants are as follows: (mean age 9.27yrs ± 3.16**,** body weight 43.98kg ± 23.99, height 133cm ± 16.36), as shown in Table 1, with a mixed gender (m=9 & F=6). Their mean initial hyperphagia behaviour was detected at 5.5yrs ± 2.5yrs. Anthropometric measurements and genotypes were also recorded. The PWS genotype delineations were

"Deletion or Non inherited" (n=9), UPD (n=3), and "Imprinting error" or "Micro deletion" (n=3) (Table 6).

Table 6 Anthropometric Characteristics

Anthropometric, behavioural and clinical characteristics of children and adolescents with PWS.

Number and Gender	PWS n = 15		Female = 6		Male = 9	
Measurements	Mean	SD	Mean	SD	Mean	SD
AGE	9.27	± 3.16	9.66	± 3.20	9.00	± 3.46
Weight	43.98	± 23.99	42.38	± 16.58	45.04	± 28.85
Height	133.13	± 16.36	137.20	± 14.63	130.23	± 18.30
Dose	1000mg = 8 750mg = 4 500mg = 2 250mg = 1		1000mg = 4 750mg = 1 500mg = 1		1000mg = 4 750mg = 3 500mg = 2 250mg = 1	
Genotype	Del. 9 UPD 3 Imp. error 2 Micro del. 1		Del. 3 UPD 1 Imp. error 1 Micro del. 1		Del. 6 UPD 2 Imp. error 1	
Country	Aust.11, N.Z.4		Aust. 5, N.Z.1		Aust. 6, N.Z. 3	
GH	n=12		n=5		n=7	
Medical indicators	n=15	Appetite behaviour	n=15	Intellectual & Psychological	n=15	
Developmental Delay	100%	Hyperphagia	60% (NA, n=1)	Psychiatric care	0	
Low sex steroids	Information NA	Slow metabolism	67% (NA, n=4)	OCD	73%	
Sleep disturbances	60% (NA n=1)	Low energy expenditure	93% (NA, n=1)	Temper tantrums	87% (NA, n=1)	
Thermo-regulation disturbances	67% (NA n=2)	Food seeking behaviour	67% (NA, n=1)	Possessive & stubborn	100%	
Higher pain threshold	67%			Hoarding	13%	
Hypotonia	100%			Skin picking	73%	
Hypopigment-ation	53% (NA, n=1)			Learning difficulties	87%	
Dental problems	60% (NA, n=1)					
Hearing impairment	0					
Vision impairment	60%					

Table 6. PWS group placebo-controlled, double blind, randomized, cross-over trial (n=15). Data are expressed as mean and standard deviation (SD). Del. = deletion, UPD = Uniparental disomy, Imp = Imprinting, Aust = Australia, N.Z =- New Zealand, GH = growth hormone, NA = not available, OCD = obsessive compulsive disorder.

The participants with PWS (n=15) were categorized as in Table 6. The subjects' body weight ranged from 20kg to 112kg and their height ranged from 105cm to 151cm. Thirteen participants were being administered GH: *norditropin simplex, humotrope* and *genotropin* and the others were on no medication. There were no other natural therapies or supplements ingested. All participants had developmental delay (100%), not all had hyperphagia (60%), though one parent declined to answer, similarly one declined to answer for minimal energy expenditure (93%), slowed metabolism (64%) - with four not answering, and had hypotonia (100%). The other maladaptive behaviours: skin picking, temper tantrums and stubborn behaviours are as per Table 6. It may have been difficult for parents to answer some of the questions as parents may be sensitive about certain individual questions for some issues i.e. skin picking. Also no parent/carers had an in-depth medical knowledge of their child's appetite regulatory hormones; such as elevated ghrelin. Low sex steroids were only acknowledged by a male participant's parent (Table 6).

Sleep disturbances were experienced but none were on a respirator, which would have excluded them from the trial. Temperature dysregulation and a higher pain threshold were seen in four cases and similarly there were four who experienced PWS dental problems due to thickened saliva. (Auditory difficulties or deafness were only seen in one requesting participant, though they were excluded due to other difficulties). All had conceptual understanding but IQ evaluation had only been taken by three participants. Only two of these were able to obtain an IQ score. Typical OCD behaviour was found in all five participants though this is recorded as four in the questionnaire; one parent said her child did not have OCD. The behaviours related to food seeking will be discussed in the results (Table 7 & section 3.2.4).

3.3.3 Subject group behaviours

The life-threatening behaviours in PWS are complex and individual though there are some common expected markers which may be generalized. These behaviours are well recognized by the parents/carers due to a history of constant attention. The families of the children and adolescents with PWS within our trial had all established controlled environments. However most agreed that their capacity for control was limited and that diminishing the appetite behaviour was a priority. It was agreed that for all it was near impossible to leave their child with PWS alone with food as is the case stated in the literature (Whittington et al., 2002). Even so when filling in the questionnaires not all the parent/carers believed their child experienced "hyperphagia", though all filled out the mean age of hyperphagia onset. Hyperphagia was

experienced by 60 % of our cohort (n=15) at baseline. One parent/carer declined to answer and the other 33% believed the drive to be milder though they agreed their child experienced hunger without the capacity for security or free access to food.

3.3.4 Results from hyperphagia questionnaires

This is the first trial of a natural supplement for the control of the hyperphagic drive and associated behaviour in children and adolescents with PWS. At baseline (n=15) the scores for either treatment (from HQA & HQE) were not significantly different though after four weeks treatment with CFE (n= 15) there was a significant reduction in the cumulative hyperphagia score during CFE treatment CFE: - 0.18 ± 0.66 as opposed to the Placebo (PLAC) – 0.03 ± 0.76 with a significant (P = 0.05) delta score as shown in Table 7.

The ANOVA repeated measure results showed no significant change in any specific question (Table 7). Though the statistical evaluation of these single questions is not recommended and sub-groupings are defined for analysis, by executing this process of determining the results for each question separately, it is possible to find out if any one question resulted in more change through CFE treatment than any other. This was not the case, though there was a trend of relief in question one: how upset does the individual get when denied food. Further groupings of behaviour, drive and severity are typically defined are as performed previously (Dykens, 2007). A significantly decreased delta score was recorded in the category of behaviour (n=15) due to CFE – 0.20 ± 0.61, PLAC 0.01 ± 0.81 (P = < 0.05), however no marked changes were observed in the drive, (P = 0.30) and severity (P = 0.54) (Table 7). In addition, results for gender showed significant reduction in the cumulative hyperphagia score during CFE treatment for the female group (n = 6), (P = 0.05) as opposed to the males which was not significant (n = 9), (P = 0.24) (Table 7). Also CFE treatment demonstrated a significant accumulative dose-related decrease in hyperphagia; with a mean decrease being observed at the highest dose of 1000mg/d (n=8) of CFE 0.28 ± 0.69, PLAC -0.04 ± 0.64 (P = 0.009). Due to small participant numbers, the lower doses (750mg, 500mg & 250mg) did not have sufficient statistical power to determine any dose related effects.

As some appetite-related behaviours were less established within the cohort, there was a large variation between participant scores. Decreases ranged up to four points per question, though due to the age of the cohort many behavioural measurements were already at the lowest rating - with no possibility of change. Therefore, some of the more severe behaviours i.e. questions

related to rummaging through the trash or night stealing (Q.4 & 5) - within the groupings of behaviour - demonstrated a strong overall change. Seeing only six of the cohort (40%) reported rummaging or taking food at night and (60%) stealing food (Q.8) these behaviours were not well established at baseline. Therefore, the results created an overall trend; simply, those with less strong behaviours experienced less change. Taking the lower baseline scores into consideration, individually CFE administration induced an accumulative easing in appetite behaviour for one third of the participants.

Table 7 Results Study One

Results for accumulative change baseline syndrome.			)articipant		-Willi
	CFE		PLAC		t-test CFE
Groupings	Mean	SD	mean	SD	P value
n =15	-0.18	±0.66	-0.03	±0.76	0.05*
1000mg (n = 8)	-0.28	±0.69	-0.04	±0.64	0.009**
750, 500 & 250mg (n = 7)	-0.09	±0.65	-0.01	±0.86	0.57
M, (n = 9)	-0.20	±0.71	-0.06	±0.92	0.24
F, (n = 6)	-0.16	±0.59	0.02	±0.44	0.05*
Behaviour (Q. 2, 4, 5, 8 & 10)	-0.20	±0.61	0.01	±0.81	< 0.05*
Drive (Q 1, 3, 6 & 9)	-0.25	±0.67	-0.12	±0.66	0.30
Severity (Q 7 & 11)	-0.13	±0.81	0.00	±0.83	0.54
HQ, Single question, ANOVA repeated measures					P value
Q. 1: How upset when denied food?					0.06 0.71
Q. 2: How often bargains, manipulates for more food?					0.38 0.88
Q. 3: Once food on mind how easy to direct away from food?					0.73 0.70
Q. 4: How often forages through trash for food?					0.37 0.22
Q. 5: How often gets up at night to seek food?					0.33 0.71
Q. 6: How persistent in asking or looking for food when told no?					0.32
Q. 7: Time spent talking about food or engaged in food behaviour					
Q. 8: How often tries to steal food?					
Q. 9: Level of distress when others stop food, talk or behaviours?					
Q.10: How clever or fast in obtaining food?					
Q. 11: Extent that food interferes with functioning daily routine?					

Table 7. PWS group placebo-controlled, double blind, randomized, cross-over trial (n=15), two tailed, paired *t*-test, baseline treatment, CFE = *Caralluma fimbriata* extract (treatment). PLAC = placebo (maltodextrin/cabbage leaf) treatment. HQ = Hyperphagia questionnaire Dykens (2007), * = significant difference from the baseline.

This thesis included the accumulative data related to individual participant's results - from Baseline to end of treatment – mainly to define any correlation with the specific features of PWS, ie. the participant's body weight or the length of time the participant has experienced the hyperphagia. Individual change due to the placebo & CFE supplementation had scores ranging from (CFE) -12 to +1 and (PLAC) -7 to +12 (Figure 8). Interestingly the participants with the most significant individual decreases - due to CFE - ranged in appetite behaviours between 60 - 72% in overall strength; defined by analysing baseline Likert scale scores statistically working out the percentage of the baseline score against the full score (100% = all 5 point scores for all 11 questions). There was a correlation with those individuals with a stronger percentage baseline score, with a dose related decrease during CFE administration Those mainly in the 1000mg/d groups within the lower body weight range i.e just over 40kg, with a higher baseline were able to decrease their score on the HQ (Figure 8). Lastly the strongest mean effect was an overall value within the whole group (n=15), recorded on the second week of treatment, with a mean decrease in hyperphagia due to CFE of (P = 0.01). This is discussed further in Section 3.3.1.

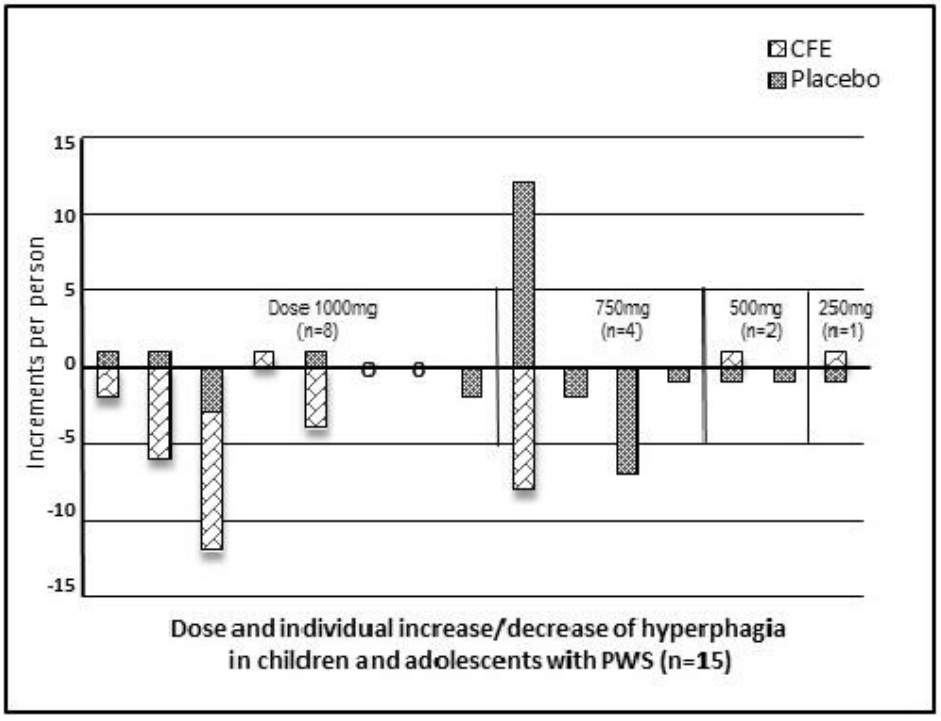

Figure 8 Individual Outcomes

Histogram of alterations (drops or rises) in appetite during the placebo-controlled, double blind, randomized cross-over pilot trial over a 10-week period investigating the effect of Caralluma fimbriata extract (CFE) vs placebo maltodextrin/cabbage leaf, in children and adolescents with PWS (n=15). Delta scores were calculated over a 4-week period for both CFE & Placebo groups.

3.2.5 Age of Onset and Variability of Hyperphagia

The two questions that did not load for statistical factor analysis were interesting in their own right. Firstly, the hyperphagia mean age of onset was earlier than the mean age of eight normally presented in literature on PWS, though the range was larger than expected (Figure 2). Within the results the hyperphagia began at a mean age of 5.5 ± 2.5yr, with a range from 2.5 to 12 years. The earlier age of onset did not determine an increased hyperphagia. Though the variability was at times strong in some participants (up to level 4 in the Likert scale) the variability was not significant in the participants. In other words, it minimally changed from questionnaire A to H. Clearly though some participants had an altered appetite, the minimal variability between their usual score from week to week, meant the changes were not evidenced by extreme changes in personality or in an unexpected manner.

The two questions that were unable to be included for statistical factor analysis were interesting in their own right. The earlier age of onset was not associated with an increased level of hyperphagia. Though the variability was at times strong in some participants (up to level four in the Likert scale) the variability was not significant in the participants. In other words, it changed minimally from questionnaire A to H. Clearly, though some participants had an altered appetite, the minimal variability between their usual score from week to week, meant the changes were not evidenced by extreme changes in mood or in an unexpected manner.

3.4 Discussion

3.4.1 Discussion on Prader-Willi syndrome appetite behaviour

Individuals with PWS typically exhibit an appetite disorder. Accordingly, study one focused on investigating an intervention for the hyperphagia and the associated problematic behaviours in individuals with PWS. This is the first trial using a natural product to attenuate the hyperphagia in individuals with PWS and the results have also been unusual, demonstrating the efficacy of CFE by decreasing the problematic behaviours associated with the hyperphagia, in a proportion of PWS children and adolescents. Importantly this study also demonstrates the tolerability of CFE supplementation in children and adolescents with PWS with no adverse effects reported at any dose over the 4-week trial period.

In such a young cohort these results present a novel approach in the management and treatment of this complex disorder. Clearly changes were more likely present in individuals on the higher doses of CFE (Figure 1) and for those presenting with more severe PWS appetite characteristics at baseline. There was also a significant result for the cumulative hyperphagia score for those in the highest dose subgroup those (n=8, 40kg -114kg body weight), ingesting (1000mg/kg). In generally the appetite reduction was more apparent in individuals with the lower body weight. As toxicity studies have indicated that CFE has a very safe profile, it may be possible to administer higher doses even in children.

It may be considered a limitation that the assessment of the hyperphagia was solely defined by non-parametric parental questionnaires. Though daily scores of hunger on Visual Analogue Scales (VAS) - before and after meals - may have generated further understanding of the cohorts' sensation of hunger, the concentration on rating hunger may have confounded the data for certain questions on the HQ, ie. time spent talking about food or engaged in food behaviour (Q7). Further, appetite tests were beyond the scope of this trial due to geographic constraints.

To support the validity of the numerical markers, "best practice" for the management of the appetite behaviour in PWS, is supervision and routine. The PWS HQ incorporates the typical close supervisory proximity and favours a constant routine because of the PWS behavioural profile. However, a parent's numerical rating of a behaviour within their child's cumulative week's scores may be skewed by an unexpected change of routine or an incidental moment of food preoccupation. So too, expectations of future occasions (e.g. school holidays or birthday parties) (Clarke et al., 2002b, Bertella et al., 2007, Dykens and Shah, 2003) may heighten the level of food concentration and on occasion alter the routine. It is therefore recommended that to improve the trial stability in the future it may be useful to create an extra question regarding unusual occurrences and introduce an individualized appetite marker as "questions 13 & 14".

Clearly during CFE administration some parents reported a noticeable drop in their personalized predictors, for example: less clock-watching and asking for breakfast, food or snacks. This easing of problem behaviour around food was not universal, but it was clearly seen in those on a higher dose at the mid to lower range in weight, evident in the 1000mg group (n=8) exhibiting a significant decrease in overall appetite behaviours (P =0.009). Further, though 100% exhibited temper tantrums at baseline, CFE was reported to ease these types of behaviours (P = <0.05). One could speculate that long term CFE administration may contribute to a less habituated

obsessive compulsive demeanour, which is supported by the nootropic and anxiolytic effect of CFE demonstrated in mice (Rajendran et al., 2010).

In regards to the trial design, the higher volatility and reduction appearing in the second week may represent a short term change related to OCD behavioural routine or a psychological effect resulting from perhaps either keenness for change by the participant or parent. The parent may also have become more aware of their child's behaviours during this first week. Further study with a larger cohort is required to elucidate the reason or perhaps a design incorporating a placebo within the first week may reduce the effect. Even if there had been a placebo effect in this design, participants were equally assigned to treatment and placebo as first or second treatment to ensure a balanced result.

The original HQ's of the participants were very individualized with extreme differences in baseline scores. Interestingly, parent's perceptions of the hyperphagic behaviours also differed strongly. Though some parents verbally defined their home environment as *"restrictive or locked",* they noted a score of 1 at baseline on some questions. This may reflect an acceptance of their child's condition or an intervention already in place and working. In comparison, another parent marked the baseline questionnaire with higher scores, yet though they recorded a decrease in the accumulative hyperphagia rating - due to CFE, their level of ritualistic food related behaviours still needed restriction. Variability in the parent's perception may be preventable in future by researchers having parents define their child's most confounding appetite marker as "question 14". This could allow comparisons through accurate personalized predictors.

It is suggested that OCD may be a contributor to the appetite behaviour seen in PWS (Clarke et al., 2002b) or that PWS neurology determines a distortion in perception (Miller et al., 2007b). Therefore the study on the nootropic and anxiolytic effect of CFE in mice (Rajendran et al.,

2010) was of interest. One could speculate that long term CFE administration may contribute to a less habituated obsessive compulsive demeanour if it were to alleviate anxiety or create a cognitive/mood enhancement, for those with PWS. Though the endocrinology and neural hypothalamic circuitry are disrupted, routine does create a sense of homeostasis in PWS. Obviously there is inadequate pharmacological treatment into adulthood, but further research needs to define if earlier supplementation may ease the earliest appetite behaviour (Goldstone et al., 2012) and further re-model brain plasticity towards a more progressive independent routine. Unfortunately as mentioned not all the parents found the time to consistently make

written observations in their journals, however parents did report reductions in their most represented obsessive 'complex individualized markers' (McAllister et al., 2011a, McAllister et al., 2011b) especially repeated questioning about the time and/or the types of food. Unfortunately, obsessive behaviours are not defined on the HQ within a single question or category. From the journals during the trial period, it was reported by some parents that CFE noticeably dropped the personalized predictors; i.e. less clock watching and asking for breakfast, food or snacks. One parent spoke of an unexpected easing in cumulative hyperphagia during the treatment of CFE as they had thought things were not too bad. The trial made them realize how used to the difficult behaviours their family had been. Accordingly, this parent recorded the strongest individual change between their two post CFE/placebo scores CFE -12 & Placebo -3 (Figure 8) with all but one score ending at 1 (individual scores not shown).

This ease around food, as a consequence of the ingestion of CFE, in the cohort, was not across the board and was mainly seen in those on a higher dose. Undeniably more research is needed before free access to food may be recommended. Some of the extreme behaviours reported within the cohort were eating of animal's food, night food-seeking, bingeing and picking or foraging through bins and other student's bags at school. Though in this cohort, CFE made an impact on the management of some of these behaviours, in general - until further research - parents will still need to take a responsible supervisory role.

Within this trial, to maintain a reliable measurement, it was understood that the parents would stay within their general routine to increase the HQ stability. Unfortunately, for those with PWS, unexpected change of routine, non-routine experiences or even expectations of future occasions (e.g. school holidays or birthday parties), may instigate temper tantrums, anxiety or OCD (Clarke et al., 2002b, Bertella et al., 2007, Dykens and Shah, 2003). These indicators or anxiety may skew the results when placed within the weekly scores on the HQ. The medical questionnaire confirmed the majority of these participants exhibited similar typical PWS behaviours (87% temper tantrums of n=14 &100% possessive or stubbornness of n=15) as shown in Table 6. These behaviours may, by extension trigger hyperphagic severity or drive and incidental moments of food preoccupation, such as the unexpected proximity of chocolate may increase a parent's numerical rating of a behaviour within their child's accumulative week's scores. (One moment increases the whole week's score). It is therefore recommended that to improve the trial stability - when utilizing this HQ as a weekly questionnaire - it may be useful

to answer an extra question regarding unusual occurrences, especially when more severe PWS hyperphagic behaviours are not well represented.

It is clear that the typical family or parent/carer wants to reduce or eliminate the restrictive requirements placed on their child with PWS. These requirements include the effort necessary for abstinence related to the proximity of food and secondly the need to choose the best nutritional food aligned with minimising caloric intake, ie. a healthy low caloric diet. This is why families create repetitive routines and this is why "Food Security" (Forster and Gourash, 2005) is considered essential. Portion size, food choice and ritualistic intake can all be confined within the OCD routine, as a comfort to those with PWS and the safety of restriction, becomes an intervention for anxiety and overweight. A controlled regimen instilled by the parents dissuades appetite behaviours, stealing, hoarding, rummaging through bins etc. The temptation is controlled and routine propels decisions. Unfortunately, this does not allow real life, outside life, or unsupervised life, to permeate the individual with PWS's experience. There is no possibility of independent living once the child, adolescent or adult leaves the routine environment. Without self-control the outside world will become life threatening.

Within the literature, improved psychological and social complexities are not addressed within the context of a need for full independence. A panel of world experts (Goldstone et al., 2008b) stated that the main task for families was the provision and support for the "possibility" of an expanded independence. They expected families to support autonomy whilst 'walking the fence' of restriction. This was done by allowing respect for choice whilst controlling the circumstances. These controls recommend limiting the spending money and monitoring all access to food, as it is expected that, in these areas, individuals with PWS, do not have the judgment to make secure healthy choices (Goldstone et al., 2008b) and that it is a "duty of care" to restrict the environment. The panel furthered their argument suggesting that restricted environments cause relief to an adult with PWS as both the temptation and motivation of food has been diminished. Yet "walking the fence" may still impede the legal requirements for the "human right" of independence. The experts conclude that power of persuasion; in regards to the acceptance of appropriate living quarters, (such as supervised group homes) is best practice.

Unfortunately, life will always impede upon this best practise as human beings are all different. Different needs and interests cannot be confined to group homes. In some cases, denying a person with PWS access to their potential may cause more anxiety or distress and by extension

more concentration on food. Certainly denying access to food may could create a rebellious need to try that type of food or it could create a stronger concentration on the food that is not allowed. It may even create a stronger compulsive or anxious response to the circumstances. A treatment would may encourage a broader range of food in smaller portions. This may even allow more independent functioning as there are less restricted access foods. Considering the results of this study and the anecdotal case study, it may be that CFE will instil at least confidence or will power in regards to the access to food and the desires of the person with PWS

In looking for any correlation to the changes seen, though speculative, dose does seem to be a likely influence on effect. The significant decrease in overall appetite behaviours, (p=0.009) evident in the 1000mg group (n=8) may deserve consideration when discussing cases where ingestion showed little or no effect. When considering more thoroughly a higher dose - related to body mass, this study could have found more significance. Responsiveness may just hinge on treatment aligned to hyperphagia or body weight. For example, one of the males within this cohort (CFE 1000mg/112.9kg body weight) may well have experienced a decrease in hyperphagia or the associated behaviours, had he been administered 1500mg the higher CFE dose, mentioned in a previous clinical trial, in obese adults (without PWS) (Lawrence and Choudhary, 2004). Similarly, the male on the lowest dose (CFE at 250gms/18.2kg body weight) may also have experienced a decrease of hyperphagia, if he had been administered a higher dose than the 250mg during the trial. Further research on dose is recommended. Also a larger cohort may address the significant contribution recorded in gender ($F=6$, $p = 0.05$).

It is obvious that only simple therapeutic techniques were utilized in this pilot, due to the unique characteristics of this genetic disorder. It is proposed that the active components within the hydroethanolic extract of *Caralluma* are the *pregnane glycosides,* which reportedly have selective serotonin reuptake inhibitor (SSRI)- like activity (Kunert et al., 2008). One proposed notion for the mechanism of hyperphagia in PWS, is that reduced serotonin (5Hydroxytryptamine; 5-HT) mediated signalling may cause decreased signalling of satiety in the brain (Dimitropoulos et al., 2000, Dykens and Shah, 2003). Limited studies on SSRIs in PWS (Selikowitz et al., 1990, Benjamin and Buot-Smith, 1993) have shown some clinical efficacy. Whether CFE exerts effect through the influence of serotonergic pathways is addressed in study two. The PWS animal study aims to define a mechanism for the action of CFE and establish the ability for CFE to change neuroanatomical markers of satiation in PWS. Other markers of appetite mentioned in "modulation of appetite" (Section 4.3) like fluid intake and

blood pressure, are all important aspects involved in appetite homeostasis. The main issue with the children with PWS was that the more one concentrates on concerns related to the physicality of appetite homeostasis, the more one reminds the individual of the reason for the study i.e. hunger. This may also be the case with adults or perhaps the concept of testing hunger may be more established. It is clear however, that in PWS the less anxiety related activities and the less changes to routine, (Forster and Gourash, 2005) the more likely you are to get an outcome you can generalize to the population of people with PWS.

It is inevitable that this study had many limitations, firstly no blood samples were collected during the trial period, due to the age and vulnerability of our cohort. In future it is important to address the relationship between PWS and blood serum levels including ghrelin, leptin, liver function, blood lipids, and glucose tolerance tests during CFE administration. Similarly, due to ethical considerations, it was unfortunate that the study had to exclude participants with more serious history of respiratory difficulties or sleep apnoea. In PWS oxygen desaturation values can really limit sleep and by extension affect appetite and appetite behaviours, in fact quality of life (Mathew, 2011, Giordano et al., 2015). Respiratory issues are common in PWS due to muscle hypotonicity and though continuous positive airway pressure (CPAP) machines can help breathing during the night, this vulnerability meant the study did not address this relationship. It would have been relevant to investigate this treatment in those with respiratory difficulties as oxygen levels and blood flow can effect appetite (Miller et al., 2007b). These restrictions also resulted in a small sample size.

Another limitation was that many of the younger children in this cohort had lower hyperphagic scores at baseline, which corresponds with the timeline of appetite behaviour in the PWS phenotype. HQ question 12, pinpointed the onset of the initial hyperphagic behaviour as (mean 5.5yrs ± 2.5yrs). There is little pharmacological treatment during the initial hyperphagic stage or for that matter into adulthood in PWS, so further research of CFE may need to determine if an earlier supplementation of CFE has any influence on the onset of the hyperphagia (Goldstone et al., 2012) and easing of the appetite behaviours into adulthood.

A further limitation was that those on other medications were excluded from the study. This meant the trial may have missed some individuals with stronger obsessional or oppositional behaviours which are normally medicated. This was a prudent decision in regards to the

vulnerability of the participants and the minimal knowledge of how CFE interacts with other medications. Even so the exclusion left many unable to participate.

Lastly there was a limitation with the standardized measurement for testing our hypothesis. The reduction in satiety in our cohort also included complex individualized markers (McAllister et al., 2011a); some noted by our parent/carers were clock watching, asking and telling, with the repeated mentioning of when, how much, or what time food will be. Other more extreme behaviours were eating animal's food and foraging in other people's school bags or cupboards at home. It is acknowledged that the measures did not fully capture the range of individual food seeking behaviours and certainly fell short of evaluating any changes in the amount of food eaten regarding appetite or impaired satiety. Even so, they did evaluate a good range of typical PWS food related behaviours.

Clearly, though the parameters were different for each participant, the rigorous compliance of those with PWS and their parents, within our cohort, demonstrate a strong commitment within the PWS community to finding an appropriate intervention.

3.4.2 Future directions

Future studies of CFE in PWS must directly address appetite severity and satiety over the larger PWS population. The investigators would hope that due to the preliminary work, in the future, it will be easier to enrol more participants to gain more power within investigations of CFE in PWS. If possible studies will allow more understanding of the differences involved in age groups and allow a greater perspective on CFE's interaction with PWS genotype. It is imperative to experiment with time lines and dose as there is evidence of a dose-dependent response in our study. Dose-dependent effects on food consumption have also been observed in an animal study (Kamalakkannan et al., 2010b).

PWS is contiguous gene syndrome with various genetic modifiers within the PWS critical region. Future work may identify corresponding genotype – weight/hyperphagia orientation or genotype - dosage variations to initiate more understanding of the full PWS phenotype. A complex future direction may be to define which aspects of appetite are known to be traits associated with a critical PWS deletion and which aspects are more generally experienced or experienced with other conditions. It would be very interesting to address hyperphagic traits

noticed in other rare conditions or syndromes and to apply our investigations of CFE to other disorders with a genetic basis. For instance, future study may want to address the genes associated with autism on chromosome fifteen and establish if any of the PWS appetite behaviours are apparent in ASD or perhaps explore overweight and obesity associated with Down Syndrome (Bell and Bhate, 1992) to determine if CFE could help adjust the body weight or behaviour in this condition. Exploration must take into consideration which treatments have worked to decrease appetite within the general public and why there may be differences in appetite due to CFE between the general public and PWS or other syndromes.

During CFE administration it will be important to evaluate mood regulation and OCD behaviours in PWS using an appropriate measure such as the Yale Brown Obsessive Compulsive Scale (Y-BOCKS). Also future trials of CFE should investigate the lipid profiles, ghrelin levels and any changes in obesity, age and CFE administration. Defining a way to quantitate leptin resistance related to BMI would be an important goal for PWS research.

Due to our participants having experienced no side effects it may be advantages to gain more statistical power within the next study by considering blood serum levels and looking at those on sex hormones or other medications. Questionnaire time lines need to be designed to minimize variables, leaving out difficult triggers such as birthdays and it may also be possible to establish extra measures of individualized markers of appetite, alongside the validated criteria.

The hyperphagia questionnaire largely measures satiety (i.e., meal cessation or how “full” or “satisfied”) and the behaviour related to this satisfaction. If future study investigates the reinforcing properties of habit related to food, researchers may be able to develop approaches which include food reward and diet preference. Investigators could research diets composed of different macronutrient content (eg. high fat vs high carbohydrate) and define a relationship between food consumed preference, enjoyment, satiety effect, nutritional needs and serotonin or dopamine signalling due to CFE. As in the pilot trial trials need to utilize a double blind, cross-over trial of an oral supplementation of CFE against the placebo of maltodextrin/cabbage leaf though it could be very interesting to add another cohort of obese and lean participants without PWS. Also the investigators feel it would be important to extend the trial to a long term trial.

As CFE, is a commercially-available supplement it could be useful to put together a survey through the PWS community to see how many have followed in the footsteps of the initial

anecdotal trial with Mia? Also a dose question would be imperative. As this thesis has only allowed treatment within the recommended dose range of 1000mg/d, it is considered important that any future work could request an amendment to the highest dose limit, through the Australian Therapeutic Goods Administration (TGA). The dose of 1500mg/d has already been studied in one trial of adults (Lawrence and Choudhary, 2004). This does may need to be considered for those over 45 kilograms.

Further study of adult ingesting CFE may be interesting through consenting participant's, guardians (parent/carer) within locked home units. People who spend so much time dealing with the behaviour of PWS will have a very clear idea of how CFE administration effects a new adult cohort. Of course plasma glucose levels, insulin, PYY, GLP-1 and the appetite markers of leptin, ghrelin and adiponectin will need to be taken. Studies which use these markers within an appetite test may be useful during CFE testing with baseline measurements taken pre and post a caloric defined meal with CFE against a PLAC.

In future it would be advantages to determine if there is any change to skin picking or to an individualized markers of repetitive appetite driven behaviour eg. Repetitive clock watching.

This may instruct if inhibition is through OCD type compulsions or just through appetite pathways. Further anthropometric measures could include a weekly weight and waist circumference chart plus statistical analyses of weight. It would be interesting to see if CFE also changes waist circumference (Astell et al., 2013) in PWS adults and children.

3.5 Conclusion

This thesis demonstrates that an extract of the Indian cactus succulent *Caralluma fimbriata*, eases hyperphagic appetite behaviour within our initial cohort of children and adolescents (n=15) with PWS. CFE administration was found to induce a significant overall easing of hyperphagia after 4-weeks of treatment compared to placebo (P = 0.05). Due to CFE supplementation there was a significant decrease in the category of behaviour (P= <0.05) recorded by the parents and similarly a significant decrease was observed after 4-weeks CFE administration in response to the highest dose (1000mg/d CFE) (n=8, P = 0.009) which was the recommended adult dose. The individual scores indicate that adjusting the dose according to

body weight to a level higher than the cap (1000mg/d) used in this study should be investigated in a larger cohort of participants with PWS.

It is important to recognize that the participants within this trial had well documented chronic behaviours of differing severity; which had not been ameliorated or modified by any previous processes. The attenuation in appetite behaviours in PWS through CFE supplementation is therefore noteworthy. Importantly this study has established the tolerability of CFE as a natural and non-invasive approach for short term treatment and management within children and adolescents with PWS. The outcomes of this study have a potential positive impact on PWS.

3.6 Conflict of interest

There are no conflicts of interest for any of the authors in this trial.

3.7 Acknowledgments

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CHAPTER FOUR

CHAPTER FOUR

LITERATURE ON THE GENETICALLY MODIFIED ANIMAL STUDIES AND CFE'S PROPOSED MECHANISM OF ACTIVITY.

4. Introduction

4.1 The animal studies

Due to CFE's favourable modulation of appetite in study one's human clinical trial (Griggs et al., 2015b). It was important to address the mechanism of appetite correction investigating the hypothesis of similar activity in animals. Firstly, chapter five documents investigations in animals which determine if there is any appetite reduction in a GM mouse model -with a deleted gene form PWS loci due to CFE and secondly chapter five and six aimed to broadly uncover a mechanism or pathway associated with the arrested appetite, within the neurology of this mouse model. The specific genetic deletion chosen from the PWS critical loci, was the *SnoRNA116.* This deletion animal model was determined to be the best choice of animal associated with the hyperphagia in PWS (Bortolin-Cavaillé and Cavaillé, Cruvinel et al., 2014, Ding et al., 2008, Ding et al., 2005, Gallagher et al., 2002, Herzog, 2012, Qi et al., 2016, Bervini and Herzog, 2013).

To fulfil the aim; the animal studies were situated in an environment where a colony could be established. Due to collaboration with the Florey Neuroscience Institute (FNI), for housing and the Garvan institute for s suitable mouse model, two studies were able to be conducted on the efficacy of CFE. Chapter five's (Study two) investigations contained seven experiments designed to determine the effects of CFE administration on the consumption of food against a placebo of maltodextrin/cabbage-leaf (PLAC) and Chapter six's study (Study three) was designed to determine if chronic administration of CFE altered the neuronal excitation.

In regards to chapter five's study, appetite suppression testing began at six weeks of age in two separate animal models the *Snord116del* and a wildtype WT control (total n=72). Administration of CFE was commenced by training the animals to eat CFE dissolved in water

with 2% saccharin (for palatability). This was mixed with gelatine and therefore - upon setting – the gelatine cubes contained sweetened CFE or PLAC. The daily food consumed - due to the treatment (two doses) or sham - and the daily weight was documented for two months. Saccharine was added to the treatments, as CFE has a bitter taste and this enabled both treatment CFE and PLAC to have a similar taste. It was also important that the animals were attracted to the treatment and that they ingested both CFE and the PLAC without significant variation.

Scientists and the FDA have agreed that an acceptable daily intake is 5 mg/kg bw (Vavasour, 1993). Mouse study groups have been administered up to 5% saccharin in their diet for up to two years with no significant differences in tumour incidences or urinary bladder pathological alterations seen between treatment and control animals. Studies however do show that >5 - 7.5% sodium saccharin may determine an increased toxic effect of the vasa recta in the kidneys, renal pelvic hyperplasia and cell damage or hepatic necrosis (Vavasour, 1993). The investigator therefore chose a low risk dose of 2% of CFE/PLAC. In regards to saccharin's effect on appetite, a review on saccharin states that in past studies where rodents were gavage fed 0, 0.5, 1 or 1.5 g/kg body weight per day of dissolved saccharin in 1 mL water, over a year, only non-significant (NS) weight-loss was seen in the 0.5g group (Vavasour, 1993). The animals consuming saccharin enhanced food, ate the same amount of food daily. Further to this urinary excretion of ingested saccharin shows it is rapidly excreted in an unchanged form. These studies seem to justify the choice of utilizing saccharin as a sweetening agent to cover the bitterness of CFE. The sweetness of the CFE treatment and saline cabbage leaf control will not change the capability for the CNS to detect glucose or lipids during the experimental protocol for appetite signalling.

Deprivation experiments were designed regarding the capacity for CFE to alter the animal's appetite, with the aim of uncovering the mechanistic pathway of the appetite activity in the brain. The first experiments were to determine if glucose and fatty acid deprivation altered the appetite signalling in the different animals; undergoing different treatment doses or PLAC and the second were designed to assess if any changes in appetite were determined due to CFE under 50% deprivation. Further in regards to retaining the nutrients needed for activity and the digestive efficacy of CFE, chapter five's study also included an experiment on faecal weight and an energy content assessment of food over a five-day period, which was altered to four days during the protocol.

In regards to deprivation and appetite it is important to understand the mechanism of action for CFE. Study two does this by defining alterations experienced by the animals along different pathways during glucose deprivation and lipoprivic feeding. The animal study experiments utilized the fact that fatty acids and glucose influence each other and are controlled by peripheral hormones, such as, insulin, leptin and ghrelin. Within this chapter the thesis introduces these hormones and the role of the hypothalamus in regards to study two (chapter five, appetite signalling experiments, and study three (chapter six's) experiments, investigating the regulation of orexigenic and anorexigenic neurotransmitters. The review also discusses the hypothesized pathway of action within the neuronal activity regarding the neurotransmitters NPY and αMSH, in regards to the appetite signalling related to appetite and thirst.

Lastly chapter five's study was designed to define if the activity of the Indian succulent plant CFE's – supposed - active constituent: the pregnane glycosides, do in fact interact as a SSRI (Figure 15). Therefore, an experiment was designed to explore CFE's interaction with appetite whilst inhibiting serotonins receptivity within a hypothalamic orexigenic appetite pathway through antagonising the 5-HT2c receptor. The antagonist SB-242084 is penetrative of the 5HT2c receptor and was tested against a saline control whilst both strains were still treated with CFE (n=72) (x 2 doses: 33mg/kg/d or 100mg/kg/d) or the PLAC.

4.2. The Snord116 mouse model

4.2.1 The rationale behind the choice of mouse model

The animal studies aiding the determination of the mechanism included four factors: animal strain, treatment, dose and appetite signalling pathways. The choice of animal strain incorporated a SnoRNA KO, making it a GM mouse model. Within the PWS genetic loci (Figure 1) SnoRNA guide chemical modifications to aide transcription. The critically deleted region has four of these genes which are involved in the splicing of mRNA (Munce et al., 2010). The deleted SnoRNA genes on chromosome 15q11.2 -13 are believed to play an important role in the appetite behaviour in PWS. Out of all the known SnoRNA's in PWS, only *Snord116* and *Snord115* are available to be investigated in rodents. Though PWS micro RNA (miRNA) encoding research is in its infancy, researchers have determined that both the human SnoRNA

SNORD115 (also known as *HBII-52*) and or *SNORD116* (also *HBII-85*) have a complex influence during the coding for 5-HT2c receptors, which are reported to be brain-specific (Schellekens et al., 2015, Canton et al., 1996).

As PWS is a contingent gene syndrome, the full physiological consequences of each altered gene is not yet known though the literature proposes the *Snord116* to be the most likely candidate pertaining to the severity of the syndrome in humans (McAllister et al., 2011a, Gallagher et al., 2002, Duker et al., 2010, Qi et al., 2016, Zieba et al., 2015). Luckily an animal with the similarly localized deletion (*snoRNA116/HBII-85* within the paternal 15q11-q13) was available within Australia. The literature names this genetic deletion as a defining instigator of the core criteria for PWS which is also determined by a human microdeletion (Sahoo et al., 2008). The *Snord116* deletion (*Snord116del*) mouse model presents similar aspects of the hyperphagia seen in humans with PWS (Qi et al., 2016). These include the capacity for appetite alterations and reduced size. The Garvan *Snord116del* has been utilized to identify how treatments interact within hypothalamic pathways before and after genetic modification.

Though the phenotype of the *Snord116del* mouse model demonstrates similar hyperphagic behaviour, unfortunately for the field of research they do not become obese. Therefore, the full consequences of an altered appetite are not demonstrated by the *Snord116del* animal model. However, unfortunately obesity is not demonstrated in any animal model for PWS perhaps due to the fact that not all the deleted human chromosome 15 genes within the PWS critical region (Figure 1), are available for a gene targeting approach within the mouse chromosome seven. It has recently been proposed that a full PWS region deletion animal model is possible through genomic targeting in a pig animal model. Until then the deleted region of PWS will be defined by utilizing single KO mouse models as in the Garvan *Snord116* deletion mouse model, recently named in a paper as the *snord116-/-* mouse model (Qi et al., 2016).

Originally it was intended that this study would also include a rodent model for the *Snord115* deletion more specifically characterized for the genes role in 5-HT2c receptor editing (Bortolin Cavaillé and Cavaillé) (Section 4.4.4). Unfortunately, all the PWS animal models representing the deletion of the *mbii-52/Snord115* are not consistently viable. At the time of study one, the continued interest for acquiring this model was registered in the USA with Jackson Laboratory to buy the *Snord115* KO animal, however, it was clear that this strain continued to exhibit 'failure to thrive" and were dying before seven weeks of age.

It is fortunate to this thesis that the literature on the characterization of the *Snord11* deletion (Bervini and Herzog, 2013, Bortolin-Cavaillé and Cavaillé, Cavaillé et al., 2000, Qi et al., 2016) name this genes involvement with both serotonin and appetite modulation through NPY and POMC which are target mechanisms for the appetite correction initially experienced in humans due to CFE (Figures 11).

4.2.2 Cre-mediated recombination

The homozygous Garvan *Snord116del* mouse model utilized in this study, was created by loxP-flanked gene segments, deleting the *Snord116/mbii-8*5 in the mouse Jackson laboratory C57BL/6 strain. The *mbii-52/Snord116* gene was inactivated through cre-mediated recombination (Schwenk et al., 1995). Site specific gene manipulation is generated by utilizing transient transfections of a 34 bp recognition (loxP) containing embryonic stem cells (ES), by flanking the genetic locus with two loxP sites. The ES contain a vector which when applied results in cre-recombination of the genetic loci, which leads to an inactivation of the targeted gene. This is then transmitted into the germline – seen in the offspring by back crossing the new generation with the wildtype. Gene inactivation is possible with the efficacy of the deletion reaching 100% (Rajewsky et al., 1996). The cre-recombination from the Jackson animals with a loxP led to no presence of the original strain in the *Snord116* germ line. Therefore, this constituted the animals as homozygous mouse models without genotyping.

Thus in the case of the study's animals - established as "available for experimentation" by a waiver from the original breeders of the C57BL/6 strain Jackson Laboratory, it was possible to utilize a very specific control for the experiments. The control chosen was the original base C57BL/6 from the same laboratory that the cre-mediated recombination *Snord 11*6 deletion animal was bred from at the Garvan. This was to give a consistency to the characterization of the WT control in the study.

Due to the clarity of this line it was possible to simply genotype the animals from their homozygous parents (male and female). This genotyping was during the animal husbandry phase of this study and coding of the *Snord116del* or WT (C57BL/6) was also maintained by a secondary visual comparison of their size whilst transferring them to single houses. Figure 9 a & b note the size difference in the animals at three weeks WT and five weeks *Snord116del*. The active *Snord116 del* animal though five weeks old; two weeks older than the WT strain is still is far shorter in length.

Visual difference between a *Snord116del* and Wild Type C57BL/6

a) b)

Figure 9 Variation in Size of Mouse Models

Study two and three animal models images present the: a) homozygous *Snord116del* mouse model at five weeks of age and b) the homozygous WT (C57BL/6) strain at three weeks of age. It is important to mention that though the Garvan *Snord116del* mouse model is a viable representative of the hyperphagia seen in humans. The animal exhibits a 5% difference in size seen above and it is known to experience the hyperphagia observed in humans with PWS during phase three of the PWS trajectory. The earlier phase phenotypes i.e. "failure to thrive" are not similar. Therefore, this mouse model was chosen due to the main AOI for investigation i.e. aligned to the appetite attenuation and the mechanism of action in the CNS

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4.3 Modulation of appetite

4.3.1 The Central nervous system

The hypothalamus, is a primary candidate for driving the body's homeostasis (Jordan et al., 2010, Quarta and Smolders, 2014, Woods et al., 1998, Schwartz et al., 2000, Seeley and Schwartz, 1999, Benoit et al., 2000, Cowley et al., 2003, Cowley, 2003, Arora, 2006, Parker and Bloom, 2012). Fuel sensing mechanisms within the CNS have the job of balancing and

integrating the body weight set point and blood glucose concentrations within ranges (lowest to highest allowable), which are defined for survival of each species (Jordan et al., 2010).

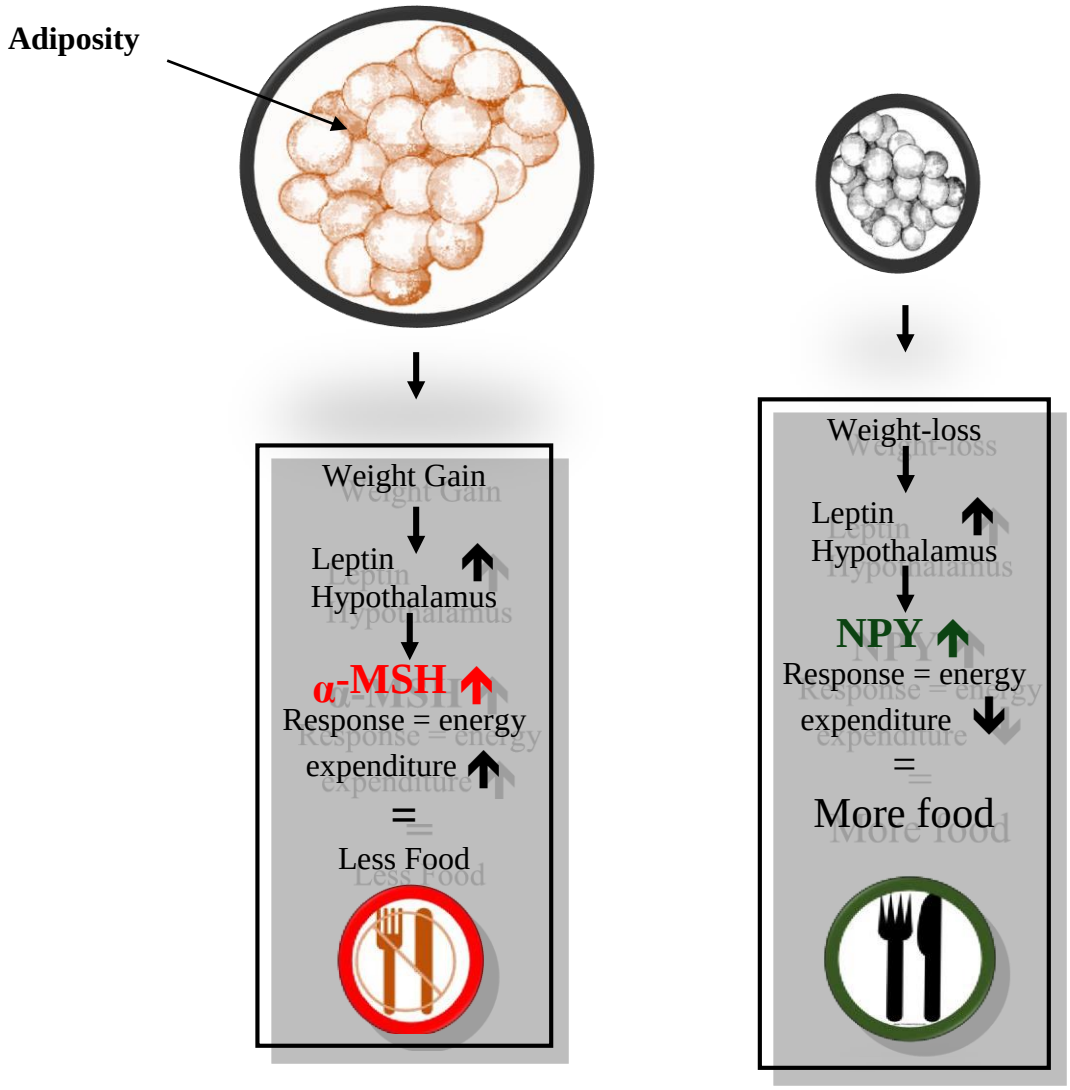


Figure 10 Adiposities' Role in Appetite

These homeostatic processes in balancing the bodies energy; for instance, the CNS glucose/insulin sensing, the brains mesolimbic system (Quarta and Smolders, 2014); leptin signals to the arcuate nucleus (ARC) of the hypothalamus from adipose tissue; oxidation of glucose (Berthoud and Levin, 2012) and other complex processes which alter appetite (Jordan et al., 2010, Farooqi and O'Rahilly, 2009, Dubern and Clement, 2012). Interestingly when body fat levels fall so do leptin levels. This therefore decreases the inhibition in the brain and increases appetite.

Neural transmitters have been identified to act within the hypothalamic circuitry as the factors which regulate changes in appetite and satiety. In regards to this stimulation: it is well-known

that energy homeostasis is mediated by a complex array of inhibitory and excitatory signals within the CNS (>50 chemical signals) (Quarta and Smolders, 2014) (Figure 3, 10, 11, 16) (leptin being one of these). The result of this cascade is a single action of regulation of either an inhibitory or excitatory nature. These neuro-hormonal massages become as succinct as regulating the portion size of food consumed (through satiety or hunger), or broad or as a feeling the need for sleep or activity (Zheng et al., 2005).

In trying to understand the mechanism of action for CFE, it was important to get an understanding of where hunger was initiated and the pathway it followed in the physiology and neurology of the mouse. Hunger is interesting as a stimulus as it can come from within the body (intrinsically) or from an experience outside the body (extrinsically), which then stimulates feeding (Hinton et al., 2004). In other words, highly palatable food can be the extraneous confounding factor when it comes to over-eating. It has been concluded that dopaminergic signalling is increased when highly palatable food stimulates the feelings of reward (Johnson and Kenny, 2010). In fact animals will go to extreme lengths to acquire pleasure (Kenny, 2011, Balcombe, 2009). For example, in regards to food, well-fed rats will seek out lollies, chocolate, peanut butter or pate' even when needing to having to enter conditions of extreme cold (-15°) to enable this action.

Intrinsic messages are experienced through increased activation in certain parts of the brain, i.e. the hypothalamus, amygdala and insula cortex and extrinsic messages (perceived from outside the body) are also experienced intrinsically in common areas of the brain and in areas specific to sensory experience. It is not clear if the conscious drive to eat from the orbitofrontal cortex (OFC) is activated due to the experience of satiety inside the body or a perception of hunger, sometimes due to reward signalling. For eg. The hormone ghrelin may signal hunger to the hypothalamus and after eating, the OFC may instruct to stop. Or... on seeing a piece of cake interest may make your OFC excite, which then could ignite ghrelin, stimulating the signal within the hypothalamus (Hinton et al., 2004). There are also limbic forebrain signals which stimulate the "liking" of food that using opioid neurotransmission will also increase the "wanting" (Berridge, 2009). This is why reward signalling is difficult to alter by will alone. It is suggested that perhaps only chemical medication, has the capacity of mediating reward regulation of the dopamine and serotonin processes within the CNS through (Kenny, 2011).

4.3.2 Analysing appetite within the CNS.

This thesis is interested in the severity, drive and the behaviour associated with the need for food investigated in study one. Though it is impossible to address these brain signals in humans, study three (chapter six) in animals utilizes fluorescent antibodies to identify these messages in the CNS. Neuroscientists measure expression of c-fos as an indirect marker of neuronal activity because c-fos is often expressed when neurons fire action potentials (Zheng et al., 2003). Marking the neuronal activity through c-fos expression and immunofluorescence has been well established (Zheng et al., 2003). In molecular biology, c-Fos is a cellular proto-oncogene belonging to the immediate early gene family of transcription factors. C-Fos has a leucine zipper DNA binding domain, and a transactivation domain at the C-terminus. Transcription of c-Fos is upregulated in response to many extracellular signals, e.g. growth factors. Additionally, phosphorylation by MAPK, PKA, PKC or cdc2 alters the activity and stability of c-Fos. Members of the Fos family dimerise with Jun to form the AP-1 transcription factor, which upregulates transcription of a diverse range of genes involved in everything from proliferation and differentiation to defence against invasion and cell damage. Within this study c-Fos is utilized as marker of neuronal activity within the CNS, to identify appetite regulating messages within the ARC of the hypothalamus and the signals relative to the melanocortin system related to satiety further downstream within the PVN of the hypothalamus (Cowley, 2003, Ellacott et al., 2006, Cone, 2005, Yosten and Samson, 2010).

The peptides and neurotransmitters involved in this study are orexigenic peptides, neuropeptide Y (NPY), which co-localize with agouti-related protein (AgRP) (not studied) and the inhibitory signals of alpha -melanocortin stimulating hormone (α-MSH) which is activated by proopiomelanocortin (POMC). Though these neuronal processes are not fully elucidated in PWS appetite, the interest in these areas is high. There is, however, much research on the hormones insulin, ghrelin and leptin (Badman and Flier, 2005, DelParigi et al., 2002, Haqq et al., 2003b, Goldstone et al., 2005, Haqq et al., 2011) which have been established to regulate the distinct opposing neuronal messages AgRP/ NPY- orexigenic and (positive need for food)

and POMC/CART as a precursor peptide of the melanocyte stimulating hormones α-MSH – anorexigenic (negative need for food) (Figure 11).

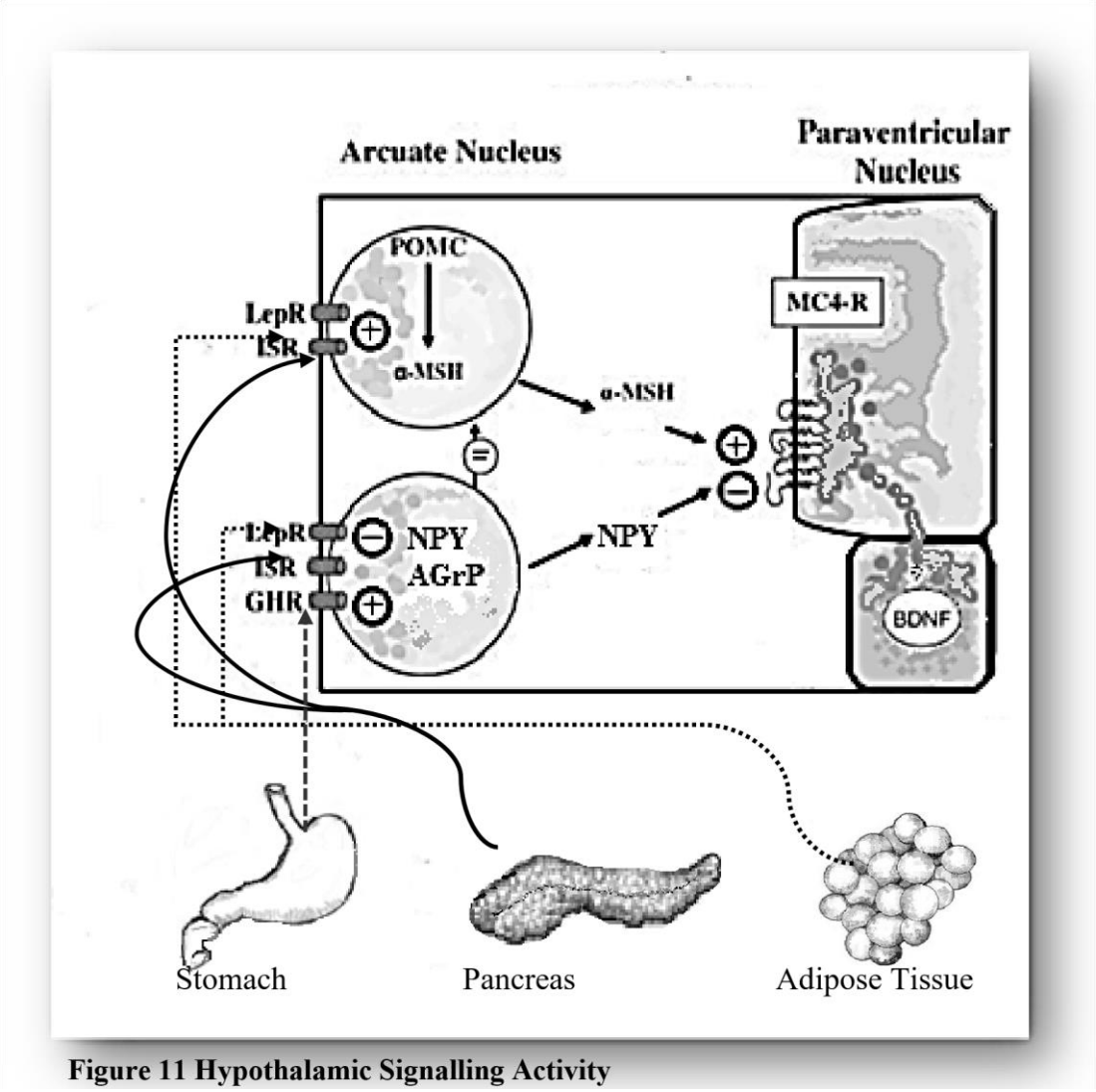


Figure 11 Hypothalamic Signalling Activity

The figure presents neuroendocrine signalling activity to the hypothalamus: stomach, pancreas and adipose tissue send endocrine signals, via ghrelin (GHR), insulin secretion receptor (ISR) and leptin receptor (LepR) to the Arcuate Nucleus (ARC), neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) which works to activate alpha - melanocortin stimulating hormone (α-MSH) and neuropeptide Y (NPY) sending messages to the paraventricular nucleus (PVN) to inhibit or excite the melanocortin receptor 4 (MC4-R), progressing to Brain-derived neurotrophic factor (BDNF).

4.3.3 Fluid consumption, and digestion in mammals.

In human – mammals - if the water content within the blood, the spinal fluid (encompassing the brain) and lymph fluid, falls, then this will need to be restored. This may be through unconscious or conscious processes i.e. by reminding the being to drink through regulation of saliva in the mouth, or by extracting water from the cells. It is difficult to address fluid intake without engaging in daily measurements. However, in studying the animals it was noticed that fluid intake did not follow the same pattern in the two strains. The study therefore measures fluid during the appetite stimulation experiments to notice how CFE altered fluid intake relative to appetite. Chapter six's study three also investigated interactions related to fluid intake when viewed through immunohistochemistry involving changes in neuronal activity within related to the ventral part of the medium preoptic (MnPO) as a representative of the lamina terminalus.

The ability to regulate food, fluid and a healthy nutritional and physical state, also involves genetics especially regarding disturbances of these systems (Fumeron, 2013). In regards to what is expected to be the results of the genetic *Snord116* deletion, the findings will not always be consistent. This is the same in humans as for example, in the case of the higher ghrelin levels: as ghrelin naturally stimulates motility, one would expect ghrelin secretion to stimulate a stronger gastric emptying in PWS along with the intake of food. Unfortunately, the life-long state of hyperghrelinemia experienced in PWS does not extend to consistent gastric emptying after food (Choe et al., 2005) in fact quite the opposite is reported. People with PWS often experience constipation (Corral et al., 2015). These results seem contradictory to what is expected by the typical evidence base. Though there may even be another logical basis to an outcome. For example as in the case of ghrelin and the lack of motility, this may be due to minimal food allowed, obesity (Kranz et al., 2012) or the hypotonic muscle tone. Therefore, chapter five investigates gastric emptying in animals in regards to the jelly treatment of CFE against a placebo in regards to metabolic function and the digestion of basic chow. Fuel or energy still accessible within the faeces will be quantified by calorimetry. This may also instruct expectations in regards to motility during ingestion of CFE in humans with PWS.

4.4 Activity within the central nervous system in animals.

4.4.1 Central nervous systems relationship to study two and three.

Within the animal system (human and mouse alike) the internal homeostatic balance is created by altering constant internal and external variables to continue the brain functioning by a metabolic fuel glucose (Mayer, 1953). The CNS is designed to regulate information or efferent signals from the parasympathetic and sympathetic nervous systems attuned to feeding or hypoglycaemic states. These feedback signals are similar for other nutrient related feedback mechanisms such as, the detection of lipids (Section 4.4.3). The gathering of the body's nutritional information converging in the hypothalamus, results in activity of neural populations as a signalling cascade. The generated chemical responses - relative to the need for fuel -control the feeding behaviour, motioning the body to seek certain nutrients (Jordan et al., 2010) or to expend the available nutrients (energy) through motion.

Study two addresses the processes within the body which aide glucostat messaging by cells. These mechanisms have interdependent components which allow metabolic set points, to be maintained within a set range of variables, by signals inhibiting or exciting other stimulus. Neuro-hormonal feedback loops reduce or increase the storage of fuel within the body due to the demand of the energy needed to keep glucose utilization available within the brain. These include utilization of energy by muscles, storage of energy in adipose tissue (fat), functioning of the gut, digestion, excretion, blood vessel flow and pressure (vasodilation or vasoconstriction), which are the effectors of heart rate. These also balance the intake of oxygen interacting with stress and emotional states both conscious and unconsciously leading to more neuro-hormonal messaging (Sherwood, 2015).

Given the complexity of these systems, which are also expected to be involved in the hyperphagia of PWS, any information on how the PWS genetic loci alters metabolic homeostasis and signalling pathways in the brain is both fascinating and extremely important. Study three investigates c-Fos activity, NPY and α-MSH within the ARC, PVN and MPOA, as representatives of the influence of neuroendocrine activity, to determine hunger and thirst in the mouse strains. Variations of signalling due to the single genetic deletion related to alterations in food consumption are important in both the neuronal signalling and in the observed physiology of the behavioural measurements. This

information is gathered during experiments triggering a feeling of deprivation of fuel which incorporates information from glucose and fatty acid utilization.

Importantly Sue Ritter and Joseph Taylor (Ritter and Taylor, 1990) have demonstrated different and distinct neuroanatomic pathways for lipoprivic and glucoprivic feeding in regards to vagal sensory neurons. As the body's homeostatic balance will try to maintain access to energy utilization by the brain necessary to survive (Section 4.4.1).

4.4.2. Glucose and circulating hormones.

Circulating hormones and nutrients are utilized by the CNS for energy (Jordan et al., 2010) when the brain's extracellular fluid is selectively separated by the permeability of the blood-brain barrier from the circulating blood (Martin et al., 1998). More than one mechanism has evolved to monitor glucose throughout the body (Thorens, 2008) as glucose is utilized by the cells to metabolise energy. Study two appetite signalling experiments - in mice -utilizes 2-DG to inhibit glycolysis. Naturally each time a glucose molecule ($C_6H_{12}O_6$,) is broken down, it becomes pyruvate: $CH_3COCOO^- + H^+$. Adenosine triphosphate (ATP) is released and so is NADH (reduced nicotinamide adenine dinucleotide); a substrate of enzymes which alters chemical groups in cells. As mentioned blood glucose concentrations dynamically help mammals regulate equilibrium. In the event of the unavailability of glycogen or blood glucose concentrations falling below - 5mM (Marty et al., 2007), an animal will immediately seek food and if food is unavailable non-carbohydrate sources will be broken down via gluconeogenesis to maintain equilibrium.

Several biosynthetic communications create the body's major source of energy: ATP production. Glucose is stored as glucagon for ATP production (Thorens, 2008) and the use and regulation of ATP create energy homeostasis in blood glucose levels throughout the body (Sherwood, 2015, MacLean and Luo, 2004b). Insulin's activity is eventually controlled by regulation of ATP-sensitive potassium (K_{ATP}) (Alejandro et al., 2009) allowing modulation through the sensing of glucose and lipids in the hypothalamus. K_{ATP} channels are expressed in most excitable tissues, playing their role of coupling intracellular energetics to electrical activity. The pancreas secretes glucagon alternately controlled by cells monitoring insulin. When blood glucose levels drop, insulin transports glucose across the cells protein membrane to prevent hyperglycaemia

(Sherwood, 2015). Glucose sensing notices changes in the concentrations of glucose within the CNS, where communication of glucose availability feeds back through the hypothalamus centrally and peripherally (figure 13).

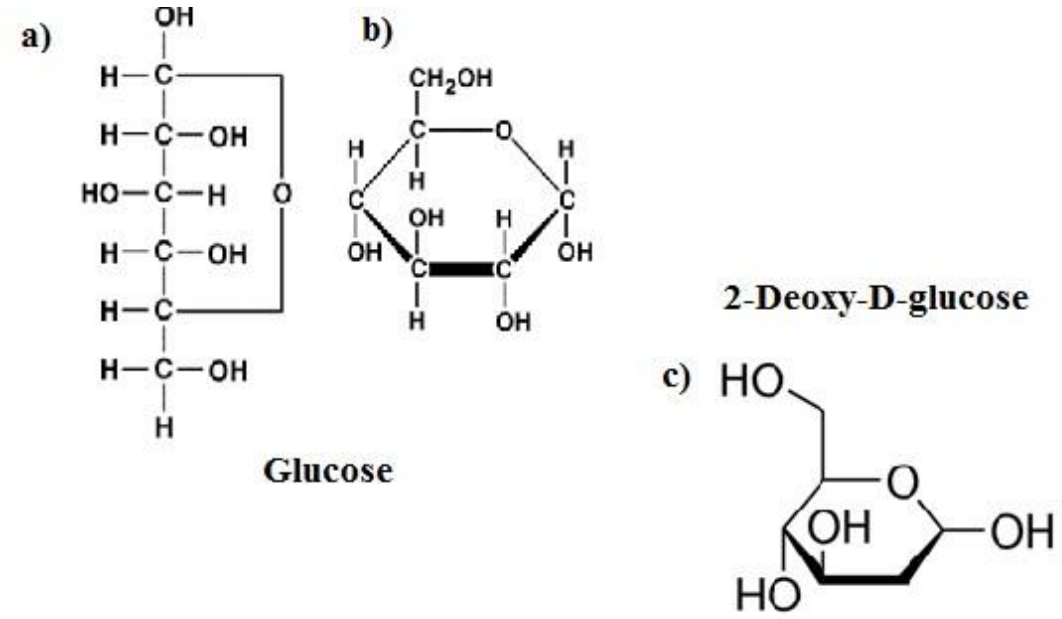


Figure 12 Glucose and Glucose Inhibition

An image of a glucose molecule a) D-glucose (dextrose), b) stable cyclic structure α-D-glucopyranose, (glucose structural form as a straight-chain and as a ring form) c) 2-Deoxy-D-glucose. C) is the Sigma image of the molecular properties of 2DG demonstrating a molecule which has been stunted of glycolysis due to the 2-hydroxyl group having been replaced by hydrogen.

Ritter's group investigated the signalling of both glucose and lipids levels in the portal vein regulating the vagal afferents through the vagal nerve. It was determined that glucose signalling follows a path from the abdomen, through the chest, neck, brain stem into the jugular and on to the medulla eventually informing the hypothalamus of the incoming glucose (Ritter and Taylor, 1990, Ritter and Dinh, 1994, Ritter et al., 1998, Ritter et al., 2000).

There is a complex process to maintain stability. Insulin is secreted from the pancreas to effectively transport glucose to the body's cells for daily survival. The cells receptors determine a reflex to food involving insulin secretion and the liver is involved in a catabolic reaction. Glucose-dependent hormones insulinotrophic peptide (GIP) and glucodin-like peptide (GLP-1) are intestinally absorbed through the epithelium (type of animal tissue) to

be sensed by the intestinal wall nerves (Sherwood, 2015, Thorens, 2008). The gut/digestive tract absorbs glucose to increase the blood's concentration and secretes glucoincretin hormones. Pancreatic *β*-cells synthesize and release or hold insulin due to positive or negative feedback relative to glucose within the blood (Sherwood, 2015, Thorens, 2008). Pancreatic α-cells produce glucagon which has a catabolic effect on energy stores. Different forms of GLUT (a glucose transporter), transports glucose throughout the body for example: GLUT -1 transports glucose across the blood/brain barrier (Sherwood, 2015). Blood glucose collects in the portal vein and is transported to the hypothalamus - along with epinephrine. Vagal afferents message glucose sensitive neurons (both glucose-excited and glucose-inhibited) of glycaemic alterations in the brains' NTS, area postrema and basolateral medulla, to further expand or reduce glucose concentrations to the hypothalamus (Thorens, 2008, Li and Ritter, 2004), where it effects the metabolism of carbohydrate, fat and protein.

Interestingly in the activity within the CNS gluco-regulation is co-distributed with epinephrine cells which suggests adrenal neural circuitry is not only combined in the body but is also coordinated in the CNS (Ritter et al., 1998, Ritter et al., 2000). When glucose deprivation is experienced in the animal then periphery feeding will be established to alter this (Mathai et al., 2004). The body will also alter its metabolic rate and temperature to conserve energy (Miselis and Epstein, 1975). In rats, insulin-induced hypoglycaemia is also found to reduce peripheral epinephrine (Sanders and Ritter, 2000).

Fos protein markers have been induced and attenuated in nuclei within the hypothalamic PVN and the adrenal medulla by 2-Deoxy-D-Glucose (2DG) (Sanders and Ritter, 2000). 2DG is a commonly utilized glucose analog (Renner et al., 2015) utilized to inhibit glycolysis in studies. During glucose deprivation - with 2DG - the action of glycolysis is phosphorylated by hexokinase to 2-DG-P, which cannot be further metabolized by phosphoglucose isomerase. This leads to the accumulation of 2-DG-P in the cell and the depletion of cellular ATP. I.p. injections of 2DG cause glucose deprivation in the brain and periphery (Miselis and Epstein, 1975), this administration will also bring on the changes in feeding, metabolism and temperature (Mathai et al., 2004) (Miselis and Epstein, 1975).

When sub-diaphragmatic vagal sensory neurons are surgically removed in rats, immediately after subcutaneous injections of 2DG (100 and 200 mg/kg) glucose utilization

is blocked (Ritter and Taylor, 1990). These results of Sue Ritter's and her experiments on injection sights in the brain indicate that glucoprivic feeding is not vagally mediated. Glucoprivic feeding is mediated through pathways in the area postrema-nucleus of the solitary tract (AP-NTS) for nutrient homeostasis and digestion.

Though the NTS has been established as the site for detection and regulation of glucose (Ritter and Taylor, 1990) another group of researchers (McDougal et al., 2013) demonstrated that hindbrain astrocytes detect glucose deprivation, which creates a stimulant for gastric motility. The pathway and mechanisms are as in Figure13.

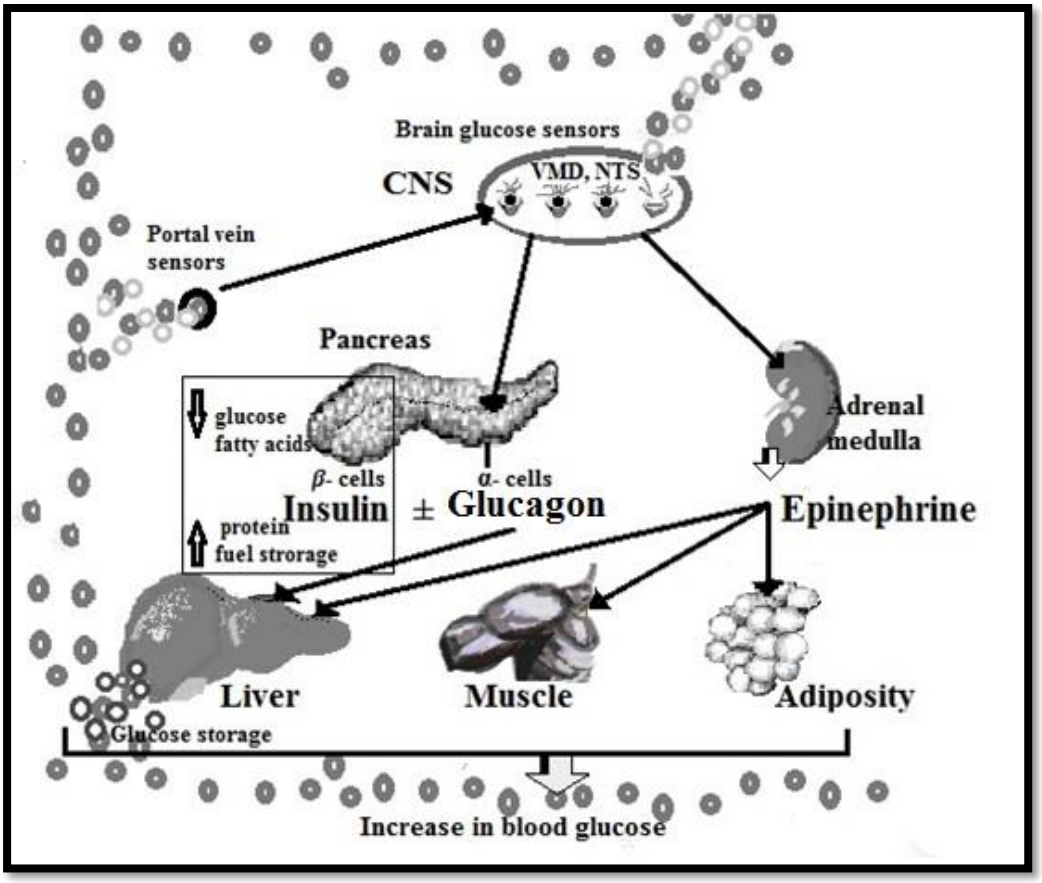


Figure 13 Blood Glucose Serum Pathway

Represents the secretions for glucose utilization. Blood glucose is transported to the CNS where sensors in the VMD and NTS interact with the adrenal medulla (a ganglion of the sympathetic nervous system) and pancreas. Pancreatic β-cells synthesize insulin interacting with positive or negative feedback to blood glucose. Pancreatic α-cells produce glucagon which has a catabolic effect on the liver. Epinephrine increases blood glucose levels controlling blood glucose, blood fatty acids and the sympathetic systems; opposite to the action of insulin.

4.4.3 Fatty acid signalling

To define the mechanism of action for CFE it is important to understand the different pathways for glucose deprivation and lipoprivic feeding. Chapter five utilizes *β*-mecaptoacetate oxidation and the interactions between fatty acids and glucose by peripheral hormones (figure). As mentioned the hormones insulin, leptin and ghrelin sense nutrients converging in the hypothalamus, and their regulation modulates NPY and POMC activating further messaging downstream appetite modulation.

Fatty acids have the capacity to become "free fatty acids' when a lipase cleaves them from triglycerides, allowing fatty acids to be utilized as fuel. When food is abundant triglyceride deposits will be stored in adipocytes. Yet as glucose sensing and lipid sensing continue in the body, if one or the other is unavailable the body will adapt. Therefore, when the primary source of fuel – glucose – is unavailable the body will utilize fatty acids as fuel instead.

Sodium mercaptoacetate (MA) blocks *β*-oxidation of fatty acids by blocking mitochondrial acyl coenzyme-A dehydrogenesis. MA is therefore able to deprive the body of utilizing fatty acids as food. Study two utilizes this reagent to explore food intake in peripheral metabolic behaviour and in pathways within the CNS (Ritter and Hutton, 1995). When the feeding activity from this blocking reagent is measured – over a four to six-hour period – significant changes are seen in the physiology of the animals. Also anatomical mediated pathways of lipoprivic feeding are able to be isolated in the CNS especially in the area postrema-nucleus of the solitary tract (APNTS).

Ritter discovered – in contrast to glucose signalling, that when sub-diaphragmatic vagal sensory neurons are surgically removed or pharmacologically blocked of fatty-acid oxidation (lipoprivic feeding) by an I.p. injection, of the fatty acid oxidation inhibitor, MA, (400 and 600 mmol/kg) and feeding is measured; mice show distinct differences between strains and relative to glucose deprivation or saline control. The capacity for long-chain fatty acids, to be oxidized occurs in the liver. Ketogenesis, the production of acetoacetate and *β*-hydroxybutyrate from oxidation is protective of the brain (Foster, 2012). This process ensures the brain continues to utilize any available glucose by transforming the freed glycerol to glucose, in the liver (Shalitin and Phillip, 2003). In regards to lipoprivic feeding the experiments involving sub-diaphragmatic vagotomy and AP-NTS lesions totally decrease lipoprivic feeding. The importance of this was that these

experiments defined different pathways to the brain for fuel utilization; showing lipid utilization was vagally mediated whereas glucose was not.

4.4.4 Serotonin

4.4.4.1 The action of serotonin

The animal study in chapter five utilized a 5-HT antagonist (SB-242084) (Kennett et al., 1997, Di Matteo et al., 2001, Lee et al., 2004, Clifton et al., 2000, Pennanen et al., 2013a), to establish if there was an interaction between CFE and the 5-HT2c receptor. Importantly study two confirms a mechanistic action of CFE.

Biochemically 5-HT is derived from "tryptophan", one of the eight essential amino acids. This amino acid is only found in the brain as it cannot be synthesized in the body. Plasma tryptophan is obtained from dietary intake and it enters the brain via the blood-brain barrier, similar to tyrosine - a competing amino acid, essential in the formation of dopamine.

Tryptophan follows a process of conversion. Firstly, it is converted to 5-hydroxytryptophan by tryptophan hydroxylase and then to 5-HT by aromatic amino acid decarboxylase. One may consider the level of tryptophan crossing the blood-brain barrier to be an indicator of serotonins availability but competing amino acids and receptor ratios within the CNS will alter the availability and activity of 5-HT. Supposedly reducing dietary levels of tryptophan can enhance impulsive behaviour and depression (Cools et al., 2011), therefore even without the genetic deletions, reduced caloric intake needed in PWS may also confound the activity of serotonin levels in the brain. Interestingly PWS children show minimal signs of depression (Lo et al., 2015). Adults do however show different levels of depression (Clarke et al., 2002a).

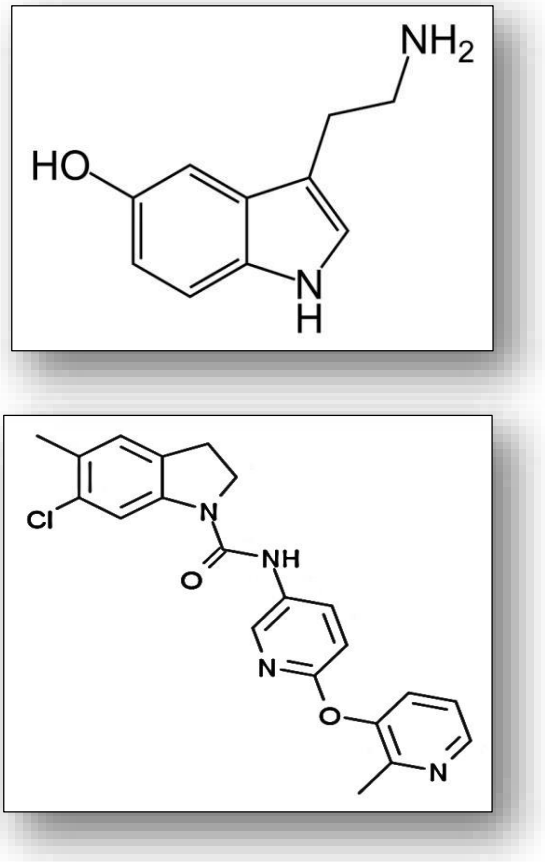
Serotonins' (Figure 14) primary role is within the gastrointestinal tract where it is metabolized by the liver. The majority of the human body's serotonin 95% (Raghupathi et al., 2013) is located in the enterochromaffin (endocrine cells within tissue) of the gastrointestinal mucosa where it is used to regulate intestinal movements (Erspamer and Asero, 1952). It is secreted from enterochromaffin entering the blood and is stored to regulate homeostasis, serving as a vasoconstrictor (blood clotting factor).

The neuropeptide 5-HT works through a large variety of G-protein-coupled receptors to control functional qualities in the body. Serotonin is secreted luminally and basolaterally which leads to increased serotonin uptake by circulating platelets and activation after stimulation, which gives increased stimulation of myenteric neurons and gastrointestinal motility.

Serotonin's 5-HT2c receptors are found in the central nervous system (CNS) (Buczko et al., 1975). This regulation is a contributor to the determination of cognitive function (Pennanen et al., 2013b) mood, sleep and appetite. Located on the cell membrane, 5-HT2c receptors activate intracellular second messenger cascades regulating anticipatory appetite and feeding – often intrinsically interacting with dopamine levels (Cedraz-Mercez et al., 2005, Di Matteo et al., 2001, Lee et al., 2004, Fletcher et al., 2009). Commonly 5-HT acts locally in neural and paracrine (cell to cell) communication (Raghupathi et al., 2013) functionally altering nearby signals depending on the tissue. 5-HT also regulates appetite reduction through communication with leptin (Yadav et al., 2011) or weight through apoptosis of adipose tissue or weight (Oh et al., 2015).

The synthesis of serotonin in specific neurons within the CNS is important to the thesis hypothesis. There are well understood pathways and receptors within the appetite homeostasis in the CNS which include downstream pathway neurotransmitter receptors Mc3 & Mc4 (Lam et al., 2007, Cowley, 2003), Npvf and Acy3 (Yadav et al., 2011). However, though these pathways are known, it is not known if they are available for appetite modulation in PWS. Also, SSRI's are used to treat compulsivity in PWS but similarly it is not known if they interact mechanistically with pathways involving appetite or even food preoccupations.

Due to the interest in SSRIs in PWS and the belief that CFE is a SSRI (Figure 15), this thesis investigated the modulation of serotonin by blocking the receptor from receiving serotonin within the CNS. Further, touching on the expected progression of appetite attenuation involving the downstream satiety pathways within the ARC and PVN (Kishore and Stamm, 2006, Heisler et al., 2006, Lam et al., 2007, Cowley, 2003, Arora, 2006, Sainsbury et al., 2002, Cone, 2005), study three determined if CFE interacted differently due to the genetic deletion associated with modulation of serotonin.



a/ Molecular structure of serotonin (5-HT).

b/ SB-242084 is a penetrative antagonist for the 5-HT2c receptor in the brain. (Kennett et al., 1997).

Figure 14 Serotonin and a 5-HT2c Antagonist SB-242084

4.4.4.2 Snord 116 deletion and serotonin

The literature on the *Snord* gene clusters within the critical PWS region describe these deletions as playing a major role in the appetite behaviours in PWS (Duker et al., 2010, Runte et al., 2005, Sahoo et al., 2008). The 5-HT2c receptor has been identified as subject to RNA editing, which is suspected to have impaired it's function in PWS (Kishore and Stamm, 2006). This is due to the altered splicing of the gene *HBII-52* - *Snord115* and *HBII-85* - *Snord116* within the critical region of PWS *(Bortolin-Cavaillé and Cavaillé).* The editing is a pre-RNA modification, not post-transcriptional as in most cases. This unusual occurrence has been identified in C/D box snoRNAs. These C/D box SnoRNAs - deleted in PWS – would normally have the job of guiding

RNA duplexes to their specific RNA targets. Their function is as a ribonucleoparticle (snoRNP) involved in biogenesis of proteins and in pseudo-uridines synthesis (Bortolin-Cavaillé and Cavaillé, Cavaillé et al., 2000).

In regards to efficacy of the 5-HT2c receptor (Bortolin-Cavaillé and Cavaillé), it is known that the deletion of *Snord115* creates an extra frame shift in the second intracellular loop of the encoded receptor, causing a change in the amino acid sequence, leading to deterioration in the efficacy of the 5-HT2c receptor in the hypothalamus in PWS. This deletion of the gene *Snord115*, pre-mRNA is subject to adenosine-to-inosine editing within five sites (Shen et al., 2013), altering the alternative splicing of exon Vb. This alteration leads to impaired functionality of the coupling action within the G-protein neurotransmitter receptor 5-HT2cR after editing in PWS. It has not been reported if this is the same device in the *Snord116.*

Though there is knowledge of impaired serotonin functioning due to the *Snord116* deletion in PWS, the question of the viability of regulation through the 5-HT2c has not been answered. It seemed plausible CFE's proposed interaction in altering the uptake of serotonin could perhaps characterize the responsiveness of the 5-HT2c receptor in the hypothalamic appetite pathway of the *Snord116del* mouse model.

The serotonin 5-HT2c receptor antagonist SB 242084 reportedly regulates PVN neural afferents through the POMC neurons and α-MSH, which are known to interact with the inhibition of appetite along the melanocortin pathway interacting with the Mc3 & Mc4 receptors, (see Figure 16) (Xu et al., 2008, Lam et al., 2007, Heisler et al., 2006). There have been studies related to receptive pathways, NPY and POMC excitation and inhibition in the *Snord116* deletion mice (Bervini and Herzog, 2013, Qi et al., 2016) but the efficacy of the 5-HT2c receptor or indeed serotonins interaction (Fletcher, 2013, Fletcher et al., 2007, Shen et al., 2013) is unexplored. By exerting an antagonist to the 5-HT2c receptor, the reception of serotonin may be blocked within this appetite system. Naturally if the pregnane glycosides in CFE enhance the amount of serotonin available for reception, antagonising the 5-HT2c receptor to the melanocortin system, will cause the mice to be hungrier. By defining CFE's regulation of appetite in these mice and then by causing hyperphagia it was hypothesized that it was possible to determine the receptor was active in the KO mouse. It was also plausible that downstream pathways were active. To add weight to this hypothesis, the opposite (hypophagia) has been demonstrated through an agonist along Mc4 receptor pathway (Lam et al., 2007). Therefore, the significance of this work is in both defining the capacity for CFE to interact with serotonin re-uptake, which was to date unknown and in identifying the capacity for genetic modulation of serotonin in the Garvan *Snord116* deletion mouse model.

4.4.4.3 Hypothesis of activity of serotonin in CFE

A supposed mechanism of effect for appetite regulation of CFE is most commonly presented through commercial claims. Sites claim that CFE works due to a SSRI effect on the hypothalamus. Though *Snord115* increased 5-HT2c pre-RNA editing is reported, the literature suggests this does not mean this receptor is without functionality in all with PWS (Kishore and Stamm, 2006). Altered regulation of this receptor has shown different levels of inefficacy in PWS (Doe et al., 2009) (Wylie et al., 2010) which may also mean different levels of regulation in downstream appetite pathways.

SSRI activity involves selective regulation in reception of serotonin by blocking serotonin uptake. Serotonin released from vesicles within the neuron is blocked of reuptake and therefore it is not able to be reused. This blocking action to the pre-synaptic neuron allows the neurotransmitter to stay in the synaptic cleft longer. Perhaps this availability allows depleted 5HT2c receptor transmitters to receive a larger amount of serotonin, demonstrated in figure 15.

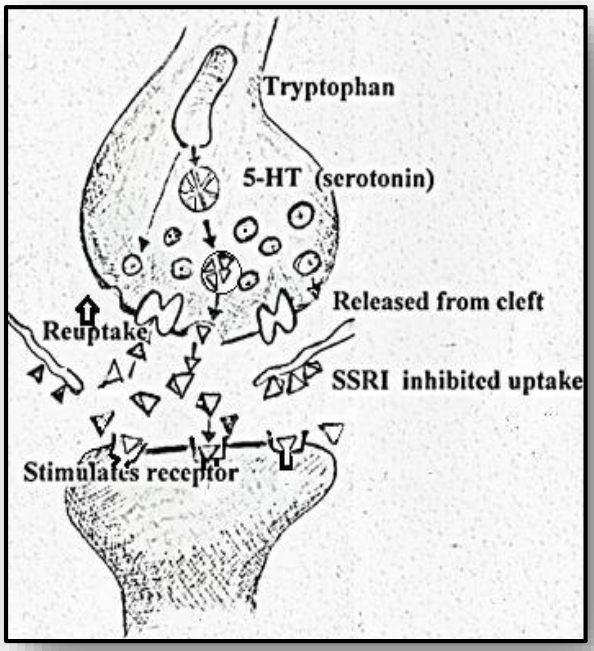


Figure 15 Selective Serotonin Reuptake Inhibiter. The figure demonstrates the neuronal interaction of 5-HT (serotonin) signalling and a selective serotonin reuptake inhibiter (SSRI). Tryptophan is transformed into the 5-HT signalling neurotransmitter which is released into the synaptic cleft between the axon of the pre-synaptic neuron and the post-synaptic neuron within the central nervous system's (CNS) hypothalamus. A SSRI blocks the reuptake of serotonin back into cleft allowing the serotonin to stimulate the receptor sending a neural signal through the post-synaptic cleft into the protein membrane.

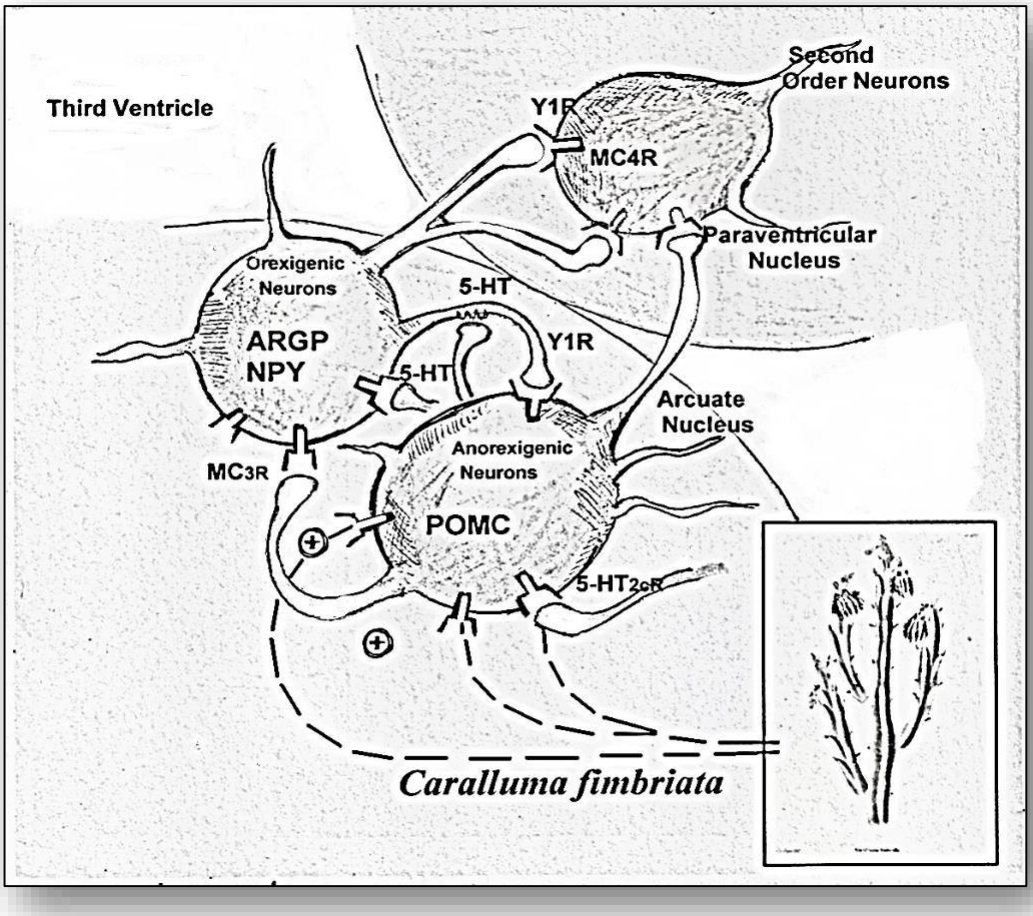


Figure 16 CFE's Proposed Pathway of Activity. The image presents the Hypothesis of the pathway for *Caralluma fimbriata* and neural orexigenic and anorexigenic interactions that portrait the neurotransmitter co-expression of 5-HT with neurons containing proopiomelanocortin (POMC). These further activate melanocortin receptors MC3 & MC4 (anorexigenic) (Lam et al., 2007) and decrease neuropeptide Y/agouti-related peptide neuronal expression (orexigenic, which results in reduced food intake (Cowley, 2003). Joanne Griggs 2011© Victoria University.

It is not known if the mechanism of uptake or serotonin receptivity is central to the development of obesity in PWS but the action of serotonin is suspected to be involved. As mentioned earlier serotonin reciprocally regulates melanocortin neurons regarding weight-loss (Heisler et al., 2006). The majority opinion attributes the complexity in the PWS hyperphagic difficulties to the brain's hypothalamic pathways and disturbances within these systems (Benelam, 2009, Holland et al., 2003). Additionally, serotonin is involved in modulating the release of other neurotransmitters including dopamine – reward pathways, glutamate and GABA, plus basal and

stress induced regulation of hypothalamic and pituitary gland hormones such as oxytocin, prolactin and vasopressin (Fulton, 2010). Fortunately, the pregnane glycosides in CFE reportedly target the endorphin and serotonin systems.

Past studies on the activity of pregnane glycosides have shown some efficacy associated with the pathways of interest. A study on the treatment *fenfluramine* (in non PWS) noted that administration activated the 5-HT2c receptors with an increased firing rate in the expression of the anorectic POMC neurons. Plant pregnane glycosides are believed to share this pathway of reciprocal regulation in the signalling of appetite (Kamalakkannan et al., 2010b) as was mentioned in Section 2.4.4 in regards to *Hoodia gordonii*. POMC 's interaction extends downstream to the melanocortin pathway, decreasing appetite (Cowley, 2003) as presented in Section 4.3.1, on the CNS. This activation inhibits the consummatory components of feeding and increases the markers of activation in brain c-Fos immunoreactivity in mice**.** To investigate and measure activity in the brains of animals in molecular biology researchers

4.4.4 Measurements of neuronal C-Fos

C-Fos is a part of a large family of transcription factors which belongs to the immediate early gene family, which means c-fos proteins are amongst the first to be expressed. In neuroscience research, neuroscientists measure expression of c-Fos as an indirect marker of neuronal activity because c-Fos is often expressed when neurons fire action potentials. It has a leucine-zipper for DNA binding and a transactivation domain at the C-terminus. Transcription of c-Fos is upregulated in response to many extracellular signals, e.g. growth factors or phosphorylation.

4.5 Aim of the animal studies.

The aim of the animal studies two and three (chapter five and six) were to determine if chronic administration of CFE altered the food intake and body weight gain in an animal model of PWS and to determine a mechanism of action of CFE. The *Snord116del* (SNO) mouse model was utilized as a representative of the hyperphagia seen in PWS, against a control the C57BL/6 wild type [SNO: n=36; WT: n=36: (100CFE/M: n=6 & F: n=6;

33CFE/M: n=6 & F: n=6; & PLAC/M: n=6 & F: n=6)] during the sixteen-week experimental period. To accomplish the aim, the experimental protocol involved a complex group of experiments utilizing conditional deprivation under basal and stimulated conditions, within a range physiological targets. The defined protocol culminated in statistical analysis of food and water appetite due to CFE administration, observational characterisation of the models appetite behaviours and visual confirmation of altered neuronal excitation of NPY and α-MSH during glucose deprivation. Further an important aim of the animal studies was the determination of a mechanism of action for the altered appetite due to CFE, utilizing the serotonin 5-HT2c receptor antagonist, SB242084.

CHAPTER FIVE

CHAPTER FIVE

BEHAVIOURAL STUDY OF CFE'S INTERACTIONS WITH APPETITE IN THE GENETICALLY MODIFIED SNORD116 DELETION MOUSE MODEL.

5. Study two

5.1 Abstract

Aim

This *Snord116del* mouse model (SNO) is utilized as a representative of the hyperphagia seen in PWS. The aim of this chapter was to determine and compare if chronic administration of CFE altered the food intake and body weight gain in the SNO mouse model against the C57BL/6 wild type (WT) control. The secondary aim was to characterize the differences in appetite behaviour determined between the mouse models, whilst investigating the mechanism of activity for appetite attenuation by CFE. To achieve this study three (this chapter) utilized a number of experiments involving conditional deprivation and appetite stimulation.

Hypothesis

It was hypothesized that chronic administration of CFE would alter food intake and body weight gain in both the *Snord116del* mouse model and the WT control and that there would be a dose response relative to the two doses of CFE (33 & 100mg/kg/d) against the placebo (PLAC) of maltodextrin/cabbage leaf. It was hypothesized that the strongest inhibition of appetite would be due to the highest dose of CFE in both strains.

Methodology

Animals were housed at the FNI, and ethics approval was gained through the Howard Florey

Institute Animal Ethics Committee (HFI AEC) under the submission numbered 14-081FINMH. The Institute Biosafety Committee (IBC) approval for Genetically Modified Organisms (GMO) was approved by a separate application through the Institute of Neuroscience and Mental Health

(FINMH) and confirmation of Animal ethics and IBC approval was gained from the Victoria University Animal Ethics Committee (VU AEC).

Characterization of appetite: by the amount of basic chow (BC) eaten daily, body weight daily and behaviour during these experimental protocols, were determined in the SNO and WT strains [SNO: n=36; WT: n=36: (100CFE/M: n=6 & F: n=6; 33CFE/M: n=6 & F: n=6; & PLAC/M: n=6 & F: n=6)] over a sixteen-week experimental period. Due to homozygote birth, genotyping was not necessary. Treatment was started at six weeks of age in all first generation animals (n=72) at either of two doses CFE (100mg/kg/d & 33mg/kg/d) or the PLAC of maltodextrin/cabbage leaf. Daily food left over (FLO) was measured every twenty-four-hour cycle, (including the dark cycle) and body weight was noted daily, over an eight-week period.

After this time a faecal analysis was made to investigate digestive absorption in all the animals, followed by an acclimatization period before experiments testing the outcomes of CFE/PLAC after administration of appetite signalling a) glucose deprivation (induced by i.p. injection of 2deoxyglucose (2DG) 400mg/kg or 10mg/25g mouse), (b) fatty acid signalling (induced by i.p. injection of beta-mercaptoacetate (MA) 100mg/kg or 2.5/25g mouse), by (c) an injection of sterile saline (SAL), as a control. Experiment d) identified the role of the 5-HT2c receptor in the appetite behaviour by an (i.p. injection of SB 242084, 1.0mg/kg), a 5-HT2cR antagonist. Lastly a four day 50% food restriction determined the capacity for CFE to alleviate appetite under fasting conditions.

Results

The results of experiment one, over the eight-week appetite experiment between strains, determined some unexpected outcomes with the SNO 100CFE group ingesting the lowest amount of food, over all groups and WT 100CFE ingesting the highest amount of food: - SNO100CFE 3.32 ± 0.67; WT-100CFE 3.59 ± 0.73, (P = 0.005). The appetite increase in the WT animals on the highest does of CFE, was most probably due to malabsorption which was determined by faecal collection and calorimetry. The WT-100CFE animals had a significant heat energy (HE) content compared to the SNO-100CFE animals: WT- 100CFE 61.75 ± 20.82mj, SNO-100CFE 26.63 ± 2.32mj, (P = <0.001)

The appetite signalling outcomes for comparisons of hunger, after four-hours due, to the appetite signalling stimulants, determined many significant difference especially due to strain. These included but were not limited to the SNO animals exhibiting hyperphagic behaviour when

administered the control SAL SNO (n=36) 1.39 ± 0.32g, against the WT 1.14 ± 0.40g, (*P* = <0.001). Outcomes were also significant during 2DG i.p. administration with strains (n=12 per group), demonstrating an opposite effect: SNO-100CFE 0.77 ± 0.37g paired with WT100CFE/kg/d, 1.19 ± 0.18g, compared to the SNO-PLAC, 100 ± 0.25g paired with SNO-PLAC, 155 ± 0.55g, (*P* = 0.02). The strongest effect in treatment was defined during SB 242804 experiments, in comparison to the control SAL and the other two stimulants, 2DG and MA. The total food intake (n=72) was: SB 242804, 1.56 ± 0.54g, against SAL, 1.14 ± 0.40g, (*P* = <0.001).

The complexity of the experimental protocol defined various interactions between deprivation which caused some unusual fluid intake measurements. The most significant were during MA stimulation which demonstrated a reduction in thirst between strain; SNO 2.44 ± 2.01ml compared to the WT 5.48 ± 1.86ml, (*P* = <0.001). This warrants further study. There were also significant differences seen in fluid intake during the deprivation study. CFE was unable to hold off appetite over the length of four days 50% deprivation.

Conclusion

Investigation of appetite behaviour in the cre-mediated recombination Garvan *Snord116* deletion animal model (SNO) from an original C57BL/6 base and a C57BL/6 WT control from the same laboratory, demonstrate that CFE did not cause a significant difference in appetite in any of the SNO and WT control experimental groups. There was no significant appetite reduction within the SNO strain due to CFE over the eight-week chronic appetite study, though a significant difference was observed due to treatment CFE between the strains with the SNO animals (n=12) demonstrating the strongest reduction in appetite. Unexpectedly the highest intake of chow measured was in the WT-CFE group at 100/kg/d (the highest dose), (*P* = 0.005). The faecal experiments determine that the increased appetite in the WT-100CFE was due to malabsorption of diet resulting in increased HE in comparison to all other groups (*P* = <0.001). Also hyperactive behaviours observed in the WT mice ingesting CFE may have increased the need for food.

The outcomes during the appetite stimulation experiments showed many significant interactions both due to CFE and strain. For example, there were significant differences during glucose deprivation 2DG i.p. administration with strains experiencing opposite effects i.e. stimulated feeding in the WT animals and reduced feeding in the SNO strain, 100CFE (*P* = <0.001), 33CFE (*P* = <0.001) and the PLAC of (*P* = 0.003). Importantly the fourth appetite stimulation

experiment utilizing the 5-HT2cR antagonist; SB 242084 (Kennett et al., 1997) ultimately identified the role of the 5-HT2c receptor in CFE induced reductions of appetite. As expected the experiment correctly enhanced feeding in all animals including blocking the natural serotonin levels in the PLAC groups. This determined SSRI activity due to CFE leading to a need for future study. Even so CFE had no capacity to adjust the animals' appetites during the four-day 50% appetite deprivation study.

In regards to water appetite unusual interactions were defined between strains and between treatment especially during the four-hour MA stimulation, with lowered water appetite in all the SNO mouse treatment groups which determined an interaction between the *Snord116* deletion and water appetite during the blocking of fatty acid oxidation.

Further different appetite behaviours, postures, adiposity and activity during the complexities of the experimental protocol demonstrated unusual activity that will enable some important characterization of this animal model related to CFE and the PWS deletion SnoRNA 116.

5.2 Introduction

5.2.1 Caralluma fimbriata appetite study in the Snord116 mouse model

CFE's expression has not been fully validated through research in animals. Questions arise as to the mechanism of action for both weight reduction and for the documented decreases in appetite, i.e. children and adolescents of study one have experienced an altered appetite due to CFE. It is important to try to understand why this reduction has occurred and how this interacts with the genomics in PWS. The Garvan *Snord116del* mouse model (*SNO*) described in Section 4.4.3.2 was envisaged as the best animal model capable of determining the capacity for CFE to alter food intake, once the hyperphagic eating behaviour had been established. Further it was hypothesized that experiments in the animals would help determine CFE's proposed activity as a SSRI. As yet this has not been validated through research in animals.

Two studies were conducted on the efficacy of CFE in mice: study two this chapter and study three, the immunohistochemistry study in chapter six. Study two involved experiments designed to determine the effects of CFE administration on the consumption of food at two separate doses (CFE 100mg/kg/d & CFE 33mg/kg/d) against the placebo of maltodextrin/cabbage-leaf. Appetite suppression testing began at six weeks of age in the two animal models (n=72), where CFE/PLAC was administered by jelly dosing. This was commenced by training the animals to eat CFE dissolved in water with 2% saccharin (for palatability), mixed with and therefore contained in gelatine cubes. The amount of basic chow (BC) eaten from the age of six weeks was documented for eight weeks. Upon completion of these measurements chronic administration was continued and faecal weight and heat energy (HE) of the excreta was determined by collection of faeces and calorimetric calculations.

After initial baseline measurements of CFE's action in SNORD vs WT animals, further appetite signalling experiments were designed to determine if glucose, fatty acid or sham deprivation altered the appetite. Further investigations determined if the 5-HT2c antagonist (SB 242084) would alter the amount eaten over four-hours administration, leading to more understanding of

CFE's interaction with this important serotonin receptor. These experiments were conducted with single housed animals when spontaneous feeding was low. Treatments were separated by two - five days and all groups maintained their earlier experimental administration from the earlier Randomization.

The methods used are well established (Mathai et al., 2004, Ritter et al., 1998, Ritter and Hutton, 1995). On the day of the experiment and at the same time for each group the food and water was removed ninety-minutes before the administration of the reagent and the mice were weighed to adjust the dose determination. At the beginning of a four-hour observation period the treatment was administered as per Appendix J a) and the animals were returned to their single houses with premeasured food. Their behaviour and intake was monitored and after four-hours measures were made of water and food intake.

Investigations determined if CFE could alter the appetite behaviour when the animal was deprived of 50% food, over an extended period of four days. The level of each animal's daily intake of chow was determined from the earlier experiment and halved as the daily allowance for a four-day period. On completion a pre-measured amount of food and fluid was offered overnight (dark cycle) and the amount left over was measured as the final ingested determinant.

5.3. Study two protocols

5.3.1 Experimental protocol

Experiment 1: was designed to determine if chronic administration of CFE altered the food intake and body weight gain in the *Snord116del* mouse model (SNO) against wild type (WT) controls through jelly-dosing of CFE.

After birth, weaning, acclimatization and jelly dose training, six groups of animals received one of two doses of CFE or basic chow group ingesting a placebo (PLAC) whilst daily food intake and body weight measurements were taken daily [(n=72) n=12 per group, SNO & WT x 2 doses CFE 33mg/100mg & SNO & WT x PLAC].

Experiment 2: investigated the differences in dietary absorption of food intake in SNO vs WT due to the differences in genotype and CFE administration by collection and examination of the faecal discharge in all animal groups.

During the 12th week of continued CFE/PLAC administration, three days of faecal discharge was collected and weighed as the accumulative output. Calculations included calorimetry of dried powdered samples for gross energy (HE) excreted per animal and animal groupings.

Experiment 3: investigated the effects of CFE on (a) glucoprivic signalling (b) fatty acid signalling and (c) by administration of a sterile saline control.

Appetite signalling tests commenced at the end of the appetite observations at 13 weeks of age (n=48), (randomized and separated by 2-5 days). CFE's effect was investigated after stimulated feeding by a) blocking glucose utilization (induced by i.p. injection of 2-deoxyglucose 400mg/kg or 10mg/25g mouse), b) by blocking fatty acid β-oxidation (induced by i.p. injection of beta-mercaptoacetate 100mg/kg or 2.5/25g mouse) c) by saline as a sham treatment, also administered by i.p. injections.

Experiment 4: investigated the potential role of the 5-HT2c receptor in CFE induced reductions of appetite over a four-hour period in the SNO and WT mice, by utilizing the d) 5HT2cR antagonist; SB 242084 (induced by i.p. injection of SB 242084 in a saline vehicle) (Kennett et al., 1997).

Experiment 5: determined the consumption of food and fluid in each group of mice [(n=72) n=12 per group, SNO & WT x 2 doses CFE 33mg/100mg & SNO & WT x PLAC] on completion of daily restriction (induced by 4 days of 50% food restriction) (Mathai et al., 2004).

5.3.2 Ethics protocol

Animal Ethics Committee (AEC) and Institute Biosafety Committee (IBC) was obtained from Melbourne University, Institute of Neuroscience and Mental Health (FINMH), and Victoria University respectively. The HFI AEC submission was numbered 14-081-FINMH and all dealings with Genetically Modified Organisms/animals came under the banner of the Research Facilitation Managers approval in an overriding approval for the institution by the IBC. The *Snord116del* mouse model was acknowledged to meet the NLRD PC1 a) criteria as per the current Gene Technology Regulations 2001. The application was therefore submitted to the Bioresources manager regarding Gene Technology and Biosafety Committee approval for the HFI containment facility. Notice of the approved dealings regarding the NLRD PC1 a) dealings in FINMH Bioresource facilities was obtained from the Bioresource Manager also on Feb. 19th 2015, before transportation.

5.3.3 Student investigator training

Training related to handling of GM organisms, administration of lab procedures and biosafety management were undertaken by the student investigator as per Appendix D. Waste disposal procedures were organized to be carried out by licensed services and storage, spills and packaging procedures were discussed with both facilities. Risk assessments were completed and all goods were packaged as per occupational Health and Safety (OH&S) training and as outlined by the relevant dangerous goods codes. The availability of protective equipment was determined through a biosafety induction at WCHRE. The lab managers and technicians were made aware that the goods were being transferred. Please find a list of the student investigators appropriate training in Appendix D.

5.4 Material and Methods

5.4.1 Animal management

5.4.1.1 Animal Ethics and transportation

The GM KO mouse model: the Garvan *Snord116del* (SNO) and the wild type (WT) control, from the original C57BL/6 strain (identified as the base homozygote strain for the recombination of the SNO mouse model at the Garvan Institute in Sydney, Australia) were utilized for the animal work at the NFI. The animal studies were situated at the FNI, Melbourne after ethics was gained through the HFI AEC under the submission numbered 14-081-FINMH.

The IBC approval for GMO's was approved by a separate application through the FINMH and confirmation of Animal ethics and IBC approval was gained through VU AEC. The transport protocol was under the category of Non-infectious Genetically Modified Microorganism & Organisms - UN 3245. An order was placed through the Garvan Institute of Medical Research animal facility on two separate occasions. Once to transport the SNO mating pairs and secondly to transport the C57BL/6 (WT) strain.

Assistance was given to the investigator by the Lab Manager/Technician & Financial services in organising the courier as the order needed the FNI Animal Facility Manager to confirm that the transporting lab facilities and animal health reports were suitable for the end-use (as

indicated in the FNI permit conditions). The transportation was organized with Road/Air link, sending the animals in a single box with ventilation and bedding.

The animals were transported to level three of the FNI animal facility a PC2 area classified as "dirty". Communication was had with the receiving house manager to establish a protocol for testing the animals. The investigators chose the process of utilizing the newborn (6-week-old minimum) during the initial quarantine breeding to determine the health status. The animals were therefore able to enter the "dirty" area of the FNI after arrangement and confirmation of health screen permits were presented from the supplier. Costs of the health screening and transportation were defined by both organizations and the Health Screen Panel seen below for the GM mouse model was released to the FNI facility by the Garvan Institutes' animal Facility Bioresources Manager.

5.4.1.2 Mating, Colony, Feeding and Animal Containment

On arrival at level three the homozygote mating pairs (Ding et al., 2008) were placed together in polycarbonate mouse cages with stainless steel wire bar lids (34cm Long x 22cm Wide x 14cm Deep, Floor Area: 216 sq. cm). The lids were designed to hold both the basic chow (BC) and a heavy duty size seven plastic water bottle with rubber stopper which tapered 3.5cm corked the bottle allowing a stainless steel watering tube, the length of 4.5cm with a control ball in the tube .4cm diameter, through the centre to control the flow of water.

To begin with consumption of both food and water was ad libitum. The houses were placed standard conditions (SC) which was a temperature-controlled (21°C) room within the PC2 animal facility with 12-h light:dark cycle (lights on 0600–1800 h).

After conception the pregnant females completed their pregnancy cycle for gestation which culminated in the birth of an arbitrary number of pups per litter. The generational offspring spreadsheet and numbers per litter may be viewed at Appendix F. Due to good numbers per litter this study was able to utilize only first generation animals from the mating pairs for the experimental protocol. No pair had more than two litters within the timeline, which therefore meant there was a good spread of first generation animals to Randomize, without too many having siblings within the same treatment groups.

Each new litter of pups stayed with their mother until they were weaned at four weeks of age. Following weaning the pups were placed with their siblings in a shared house. A small portion of cereal was placed in a dish to help feed any of the weaker animals. The larger litters were also separated into male and female groups after three days, ready for handling. Once the viability of the animals was assured by visual assessment of their continued growth and health they were ready for handling.

After separation from their mothers, during week five, the pups were acclimatized for experiments. This involved the pups being handled and weighed by the investigator, sorted into groups and randomized for weight, parentage and gender. Then before the phenotype timeline for hyperphagia (Ding et al., 2008) the end of week five, the *Snord116del* mice and the WT controls were transferred from the breeding room into a separate room for the experimental protocol where they were single housed in SC. Any animals not utilized from the litters were also held and maintained by the facility attendant plus the mating pairs were separated from their partners and held within the breeding room under SC until completion of study two and three. Appendix F. presents the breeding pairs and their litter numbers. The animals were consistently housed in SC and were randomly rotated over the five tier shelves - so all received similar amounts of overhead lighting. The only environmental enrichment for the animals was a small lid/dish placed at the end of the cage for their daily jelly-dosing of treatment CFE or PLAC.

5.4.1.3 Feeding the Animals with Basic Chow

Food intake was defined as a marker to help establish any switch in the animals from the early feeding to the hyperphagia. The mice were fed on a standard commercial rodent diet, called basic chow (BC) (Barastoc, custom mixed ration; Ridley Agri Products) in this thesis.

Before the treatment cycle the single-housed animals received BC ad libitum, over and above their daily supply until the four-day protocol: training for jelly-dosing was completed (within week six). After this the treatment schedule began and the daily BC was specifically weighed for calculation of the animal's daily consumption of food over a 24hr/day & night cycle. This period included uninterrupted feeding over the dark cycle. with any left-over food being weighed between 13.00 and 15.00 hrs. On average this was when the mice were asleep.

5.4.2 Animals study procedures

5.4.2.1 Groups and Coding

The 72 mice (36 male (M) and 36 female (F)), bred within seven weeks of each other were divided into 5 groups of 12, defined by allocating pups from similar parentage into separate groups whilst balancing body weights and gender. The mice were coded for identification and varied administration of the treatment. The coding utilized the categories for strain, SNO and WT, gender and dose 33/100CFE or PLAC.

5.4.2.2 Acclimatizing the animals

After weaning the mice were acclimatized daily to handling between 13.00 - 15.00pm during the light cycle, for three days. Therefore, the first handling and restraining accomplished by the investigator (apart from the earlier mouse handling training) was when the mice were handled and weighed for coding. During this the mice were identified for groups (by spreading weight, gender and the parentage randomly but evenly between the groups).

Before any new condition i.e. single caging, jelly-dosing or treatment, and before the intraperitoneal injections (IP), the protocol introduced an acclimatization process into the daily husbandry tasks of the colony. All training for treatment for feeding was practised during the jelly dosing training leading up to the treatment and for IP on weeks thirteen-sixteen leading up to the signalling/deprivation protocol. These acclimatizing days were conjoined with any protocol that was being administered at the time but they also included new acclimatizing practises i.e. restraint scruffing of the animals for two days leading up to IP (there was no acclimatisation of the actual injection).

On the fourth day of handling the mice were singly housed, tagged and recorded within their groups. From the beginning of week five to the end of all the treatment protocols - including during the jelly-dosing protocol - the mice were handled daily, wearing single layered synthetic hypo-allergenic gloves. On the first time the mice were given time to investigate the smell of the latex gloves and were lifted by mid tail in a calm and firm way restricted to one individual at a time. Any transferring from cage to cage, e.g. when their house was cleaned, was by handling in the same way. At times, however, some of the SNO animals were able to sit on the investigators hand without restraint. At all times jerky movements were avoided.

The process of acclimatization for i.p. injections did not involve actual acclimatizing to injections. The process mainly involved maintaining a calm animal leading up to the procedures and a comfort with scruffing. This was practised daily for two days leading up to the experiments, allowed for confident handling during the rigors of the appetite signalling experiments and before the perfusion protocol.

5.4.2.3 Treatment method in rodents.

As per the clinical trial, the CFE treatment was supplied by Gencor Pacific International Ltd. (Hong Kong). The constituents of *Caralluma fimbriata* (the supplement) were extracted from the aerial parts of the plant with an alcohol solution, granulated, ground and then dried. For the animal study the powdered extract was couriered from Gencor by mail enclosed in a sealed plastic bag which was able to be stored at room temperature. The powdered extract was weighed into 500mg units on laboratory scales (Denver instruments Co. 5 Orville Dr. Bohemia, NY 11716) and enclosed in lockable treatment capsules.

The inactive placebo of 200mg *maltodextrin* (glucose polymers) and 50mg *cabbage leaf* – to once again match CFE's colour and the bitter taste – had been supplied by Gencor in capsules for the human trial. For the placebo a single 250mg capsule was opened and the powdered extract was dissolved in gelatine, as per the CFE treatment

5.4.3 Jelly Dosing

5.4.3.1 Jelly Dosing Treatment Protocol

The jelly-dosing method was proven in this animal model as able to deliver the treatment or placebo to the mice in a voluntarily manner (Zhang, 2011). Though for the mice to volunteer to eat the organic flavoured CFE/PLAC it was still imperative that saccharine be used as a masking agent to help the palatability.

All the animals received jelly training at the beginning of week six for four days, without fasting. At first the jelly was given without any treatment over a three-day period. On day

four the jelly contained the placebo to add a bitter taste to the mix and then on day five the mice were treated with either the CFE or PLAC.

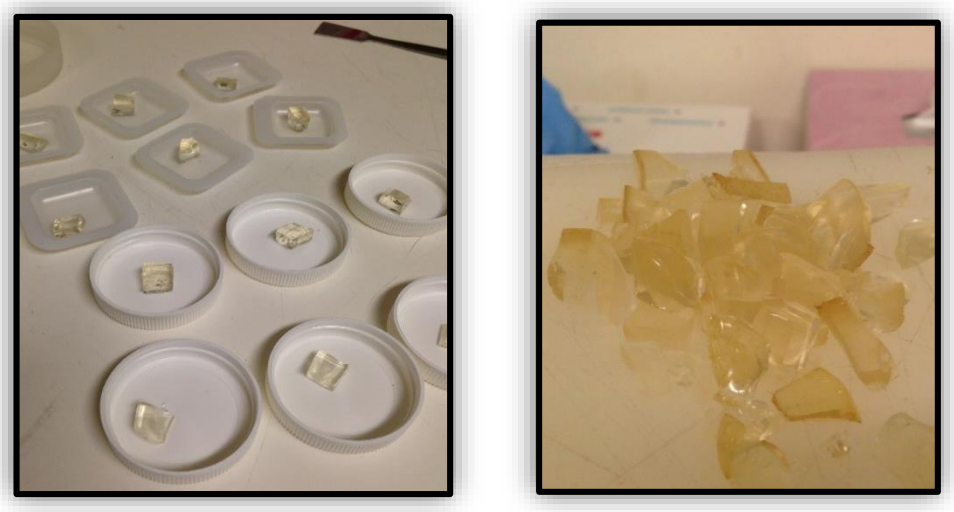


Figure 17 Jelly Dosing

The figure demonstrates the difference in colour between the jelly placebo and the CFE, plus the plating for all the treatments.

Within the jelly, a fluid form of saccharine was utilized for sweetening. The product called Low Calorie Sweetener (250ml liquid sweetener) was a heat stable dietary additive. The saccharin was administered at a dose of 2% (0.05mg = 2%), dissolved within gelatine with either the CFE or PLAC also mixed in plain tap water. For all treatments, it was expected that saccharin; which is a non-sucrose product - would not change the parameters of the study but instead it would go some way to balancing the bitter taste of the treatments, providing sweetness without adding caloric content.

To make up the treatment for the mice, one of two doses of standardized CFE powdered extract (100CFE/kg or 33mg/kg) or the PLAC (200mg/kg maltodextrin & 50mg cabbage leaf) was dissolved in 25ml of water. The dissolved mixture was then added to the dissolved gelatine in water (100ml with 2% saccharin), as described in Appendix G. This mixture was then set in trays ready to be measured at a dose for weight portion for voluntary ingestion in each allocated mouse.

5.4.3.2 Jelly-dosing treatment and placebo protocol.

All treatment procedures were conducted in accordance with AEC approval and appropriate guidelines. The vehicle for treatment *Caralluma fimbriata* extract (CFE) and PLAC was the soft jelly as described below and in Appendix G. introduced on a small plate (bottle lid) (Figure 17).

To determine the dose per mouse the protocol utilized a 12 well ice tray with a silicone base to set the jelly for slicing. A report of a dose response in mice from CFE administration at 25mg/k, 50mg/kg & 100mg/kg (Kamalakkannan et al., 2010b) helped define the dose for study two. This thesis study reduced the numbers of animals - as required by ethics - by utilizing two doses

(33 and 100mg/kg/d) of CFE related to this Kamalakkannan range

The mice ranged from 13g to 27g (over the experimental period). Considering the amount of water/H_2O normally ingested by a mouse within this range, per day (25g approx. 2ml/day). Therefore, the volume of fluid set in the jelly needed to sit within the lower range of this fluid intake; to maintain the need for water in the animals. It was also important the volume of the jelly (treatment) did not over-shoot the capacity for ingestion in the animals as then this would stop the animals eating BC.

For clarity, instead of distributing and weighing amounts of CFE from a single capsule, it seemed sensible to make the jelly using both the CFE 500mg capsule and the volume of jelly and water defined on the packaging instructions (125mls water/per sachet). This meant there was 500mg of CFE distributed evenly over a large tray of jelly which could be cut into specific edible portions per animal. When first measuring the 125ml of dissolved mixture the amount filled 13 wells, set to the apron of the 12 well tray.

$$\frac{\text{1 x CFE - 500mg}}{\text{1 x sachet of the jelly powder/ per 125ml water}} = 13 \text{ wells}$$

To enable the volume of jelly given to be the same per weight for both doses CFE 100mg & 33mg/kg, the treatment was first dissolved in a different amount of fluid before being added to 100mls of the 125mls gelatine. The 100mg/k/d was defined by mixing the 500mg capsule in 25mls H_2O and the 33mg/kg/d was also a 500ng capsule but this was mixed in 75mls of which

only 25mls was dissolved into the gelatine. This enabled less variability to the amount of jelly consumed per mouse and it also meant that any differences in appetite were not due to the portion of gelatine. Appendix G, Table 26 presents the calculations for dosage and jelly volume to the nearest dose.

5.4.3.3 Animal Jelly-training five-day protocol:

Day 1 & 2: The animals were offered the already dissolved and untreated gelatine (no CFE/PLAC) at a volume of 0.3mg in a small jar lid (as a plate) (Figure 18). The cut pieces were introduced by placing the dish at the end of cage floor away from the other food and leaving the mouse un-disturbed. The dish in the rodent house was left eaten or uneaten until the next day's weighing. Confirmation of the animal eating the jelly was made by watching the animal for 10-20min/day after offering the jelly and by searching the cage the next day to for any hidden pieces of hardened uneaten jelly in ground cover (Zhang, 2011). During study two all the animals ate the jelly over the first 24-hour period.

Day 3: The jelly was given at a volume of 0.3mg PLAC (dissolved cabbage leaf and maltodextrin with 2% saccharin) Appendix G 1 & 2. All the animals ate the jelly within 5 minutes of offering.

Day 4: All the mice received a PLAC dose at a volume defined by the animal's weight on the day. This also resembled the bitterness of CFE.

Day 5: Treatment was given as per the groupings defined by randomized coding (1 x 2 doses of standardized CFE or PLAC with 2% saccharine dissolved in H_2O). From this point observation confirmed the treatment was ingested fully for a further ten weeks as per Table 28 (7wks x appetite/weight; 3 days x faecal collection; 2wks x appetite signalling trials & 2 days IHC).

Once the voluntarily ingesting of the jelly was confirmed, a formal acknowledgement of the use of Protocol A (voluntary feeding/not gavage) was sent to the AEC of the FNI. Therefore, this was amended within the ethics application.

5.4.4 Faecal deposits

5.4.4.1 Experiment protocol

A question arose related to the hypothesis that CFE's pregnane glycosides decreased motility (Komarnytsky et al., 2013a). To determine to what extent CFE decreased or increased gastric emptying in the mice, investigations needed to define what role the *Snord116 d*eletion had in gastric emptying and if the energy from the diet eaten was in fact expended, retained or eliminated in the mouse models. This involved calorimetric measurements of the faeces. Calorimetric investigations were a precise way to define if any of the perceived differences in the animal's weight or appetite were due to how the diet was utilized i.e. through movement or warmth, without behavioural exercise experiments (Terpstra et al., 2002). Exercise experiments were of interest but they would have altered the outcome of our appetite experiments. Importantly collected faecal deposits could be analysed for gross heat energy (HE) without any adjustment to the daily routine.

Therefore, study two also involved experiments to determine the weight of the excretion -over three days - and then it investigated the excreta for HE through a calorimeter. The HE was statistically defined as a figure for the energy left within the dried, powdered faeces of the animals. Also in rodents the orexigenic hormone ghrelin has been established to increase motility (Charoenthongtrakul et al., 2009). Though this study had not defined the ghrelin levels in the animals the different faecal HE content of the SNO & WT (CFE/PLAC) determined if further research on ghrelin levels was warranted.

In summary the time-line for the excretion measurement period was three days at 13 weeks of age in all 72 mice (*Snord116del* and WT). As per usual the animals were individually housed and were ingesting their allocated treatment within SC. Even though the trial of weight and food consumption had finished all the animals still maintained their usual chronic ingestion of the treatment. The content of the collection was all the faeces deposited for each animal over the full period. The total faecal output for each animal was collected, weighed dried and ground into a powder and was placed in capsules for burning. Mean and SD of the HE content was investigated for significance through a Calorimeter CAL2K-1 (Digital Data Systems Ltd (DDS), AA-200) taken from two samples per animal in each treatment group

5.4.4.2 Faecal collection

The collection of three days' worth of faecal deposits was on the last day of the thirteenth week of the animals' life; after eight weeks of the CFE/PLAC ingestion. The investigator firstly provided each animal with a clean cage and reduced bedding of wood shavings and shredded paper. Ninety-six hours later, these cages were removed, and new cages were provided. Every dropping collected over exactly ninety-six-hours was carefully separated by sifting through the soft weighted ground cover and tissue bedding, with careful rolling action of the mass content that left all dirt and ground cover behind due to its lighter weight. The separated deposits were then stored in plastic containers and frozen in a -40c freezer till three weeks after all groups had finished the full protocol.

At the end of the three weeks each animal's sample was carefully transferred from the freezer; making sure no content was lost. This was placed in heat proof glass containers and dried in the laboratory oven at 80° for one and a half hours. After drying and before analysis by Calorimeter CAL2K-1 (Digital Data Systems Ltd (DDS), AA-200), the weight of each animal's full sample was calculated. After this, a small amount of each animal's deposit (Approx.245mg) was ground to a powder - with a mortar and pestle - and enclosed in a locked 250mg vegetable capsule. The capsule was then placed in a ladled container for weighing and calculation through Bomb Calorimetry.

5.4.4.3 Faecal energy analysis

Dietary efficiency for each animal and the treatment/animal groups was examined by calculating the HE content of the combusted capsuled faecal deposits in the chamber of a Bomb Calorimeter CAL2K-1 DDS. The chamber utilized pressurized oxygen (typically at 30atm) and electricity to ignite the weighed mass sample in a small fixed amount of water within the internal atmosphere. No gases escaped during the reaction and HE released during the sample combustion was internally measured with a thermometer. This reaction was the measurement recorded for each sample. A small correction was automatically made by the machine to account for the electrical energy input and the burning fuse.

Each separately labelled capsule was weighed for the mass of the capsule on a tared balance and placed in the metal cap with a cotton fuse for igniting. The fuse was tied around a wire which was suspended above the sample within the cylinder. The fuse dangled down to jut up against

the capsule and a tab of benzoic acid (0.05g, 10mmin diameter and 5mm thick). The benzoic acid tablet was calculated to have a specific energy combustion of 26441.2j/g per tablet.

The inner metal cap was carefully loaded (one sample at a time) and sealed in the cylinder's bomb vessel. The vessel was then pressurized to 3000KPa oxygen at a temperature of 22° before being placed into the ignition chamber. The HE content was calculated on firing in the calibrated CAL2K-4-B by calculations of the initial and absolute pressure during the energy combustion process for each sample. This was defined by the machine's in-built computer. After release the cylinder was cooled by cold water whilst the next sample was made ready to be fired. Visual confirmation of ignition was made after the oxygen was released from the cooled cylinder and the vessel was opened for cleaning. The core for each sample was documented for statistical analysis.

5.4.5 Appetite signalling

5.4.5.1 Appetite signalling experiments

At the end of thirteenth-week - after all daily food, weight charts and faecal experiments had been completed – the appetite signalling experiment began. These determined the effects on appetite, of CFE in the *Snord116del* and WT control during deprivation, utilizing a stimulant or antagonist. The Protocol A (below) reagents were: a) 2DG, glucoprivic signalling (induced by i.p. injection of 2-deoxyglucose 400mg/kg or 10mg/25g mouse) and (b) MA, fatty acid signalling (induced by i.p. injection of beta-mercaptoacetate 100mg/kg or 2.5/25g mouse), c) SAL, as a control vehicle of sterile saline and d) SB 242804, for interrupting the serotonin signal expressed on the 5-HT2cR (induced by i.p. injection of the antagonist SB 242084 and similarly deprivation study (experiment 5). The appetite of the animals was studied after four hours, moving from the light cycle to the first hour of the dark cycle. Protocol B was initiative e) 50% deprivation by food restriction defined in ethics as five days of 50% food. The last experiment was altered from five to four days during the first groups recorded observations, due to animal distress.

Study two utilized 2DG and MA to invoke counter-regulatory responses in the animals. The reagents SIGMA's 2-Deoxy-D-glucose Section 4.4.1 for glucoprivic feeding and the beta-

adrenergic-mediated inhibition of feeding by beta - mercaptoacetate Section 4.4.2were used for investigating the hypothesis that these stimulants would increase appetite in the animals and the treatment CFE and the genetic deletion would interact with this altered appetite. Experimental agent c) saline was the control for all the experiments leading to an opportunity to assess one deprivation against another. The stimulant appetite test for d) serotonin signalling, was incorporated as serotonin is strongly implicated in PWS. This test examined the association between the *Snord116del*, CFE and the 5-HT2c receptor utilizing an SB 242084 antagonist, specifically targeting this receptors activity. The antagonist in this case was not a stimulant but instead was designed to inhibit signalling of serotonin, known to be exhibited anappetite pathways within the hypothalamus of the brain (Kennett et al., 1997, Lam et al., 2007). As outlined in Section 4.4.3 this pathway was believed to produce a reduction in appetite, so by antagonising the receptor (stopping the chemical signal) it was expected that any activity of enhanced serotonin mediation by CFE (through this receptor) would be altered.

Prior to the appetite investigations - within the week of faecal experiments - night & day, all single housed animals received a daily allowance of food for ad libitum feeding. This enabled less stress during appetite signalling tests. Protocol A followed the same steps for each of the four appetite signalling tests: a, b, c, & d. Protocol B for e) 50% - food restriction varied from B. All treatments were separated between two to five days and each group's tests followed the same timeline within the study two protocol as per Appendix H Table 28.

5.4.5.2 Protocol A: appetite stimulation

All experiments were conducted with the same single housed animals, after the chronic feeding and faecal deposit tests were completed when they reached the age of fourteen weeks (n=8-19). Each of these animals were still ingesting the same treatment as per their chronic feeding tests (n=72).

The timeline for the tests incorporated two hours of fasting and four hours of observations, continuing the observations into one hour of the dark cycle, where natural feeding was expected.

The animal's morning weight recording defined the treatment and reagent injection volume as per Table 8. Observations and measurements of food and water followed an identical ordered cycle, as per the treatment and administration of the reagent (approx. one hour). The animals'

preparation for the experiment - allocated, b, d or d as per the time-table Table 28, Appendix, H - required the animals to continue in single housing with food and water were removed close as possible to 13.30pm. The animals were given their weighed daily treatment CFE x 2 doses or PLAC as per their groupings, left in a dish in the cage for voluntary administration.

The experimental food (approximately 12g for each cage) and water volume was weighed recorded and set aside ready for each animal to be placed in the cage after stimulant administration for the four-hour test. The stimulants contents were removed from the packaging, weighed on a Mettler PM4800 Delta Range scale and the solution was made up to the volume determined by the percentage of treatment, at the dose defined in the ethics application.

Table 8 Dose of Stimulants

Appetite signalling stimulant dose table for intraperitoneal injection		
Dose for 2DG	Dose for MA	SB 242084
400mg/kg	100mg/kg	1mg/kg
40mg/100g	10mg/100g	0.1mg/100g
4mg/10g	1mg/10g	.01mg/10g
10mg = 25g mouse	2.5mg = 25g mouse	.025mg= 25g mouse

Table 8. Defines the dose per animal for each experimental protocol, with a progressive reduction of measurement made to dissolve the reagent in saline as per the vehicle. 2DG,
i.p. injection of 2-deoxyglucose; MA, i.p. injection of beta-mercaptoacetate, SB 242804,
i.p. injection of the antagonist SB 242084.

5.4.5.3 Protocol B: 50% deprivation

All experiments were conducted with the same single housed animals, after the appetite signalling experiments were completed. The animals from different treatment groups were allocated for 50% deprivation in the same groups as the last three experiments (n=8-19). Each of these animals were still ingesting the same treatment as per their chronic feeding tests (n=72). The timeline for the measurements of food was after four days, though the animals weight was measured daily along with a health check. On the morning of the experimental protocol the single housed animals' weights were recorded to define the treatment dose. The dose was

administered at 50% volume of the over-all mean daily amount per animal (between 1.5g – 1.9g), defined from the chronic treatment's. After four days of 50% deprivation, each animal 's level of appetite and thirst was determined by providing the animals with a large portion of premeasured chow and bottled water. The FLO was weighed the next day after 24 hrs feeding including dark cycle feeding.

5.4.6 Measurements

5.4.6.1 Testing the Hypothesis

Measurements of weight and food intake were designed to help establish any differences between the energy intake of the different groups and to determine if and when the *Snord116del* animals demonstrated hyperphagia. The daily measurements occurred during a specific timeline as outlined in table. The design was to characterize appetite ingestion and reduced feeding and to define any change to appetite. The complexity was related to the questions below:

- Does the treatment CFE alter the outcome for food consumed over four hours, against the Placebo overall?
- Does the dose of treatment CFE alter the outcome of appetite - food consumed over four hours - against the Placebo?
- Does the strain of mouse alter the outcome of appetite - food consumed over four hours?
- Does the strain of mouse alter the outcome of CFE's effect on the food consumed over four hours?
- Does the specific appetite signalling stimulants alter the outcome of each of the above against the control (saline) within the strain and/or between the strains?
- Does CFE alter the outcome of water consumed over four hours against the Placebo?
- Does the dose CFE alter the outcome of water consumed over four hours against the Placebo?

- Does the strain of mouse alter the outcome of the water consumed over four hours?
- Does the strain of mouse alter the outcome of CFE's effect on the water consumed over four hours?
- Does each specific appetite signalling stimulant alter the outcome of each of the above against the control within strains and/or between the strains?

The experimental protocol and measurements followed a timeline where meant each animal followed the same process. The timeline is defined in Table 9, below.

Table 9 Study Two Animal Groups

Groups, numbers and timeline of study two's experimental protocol.

WEEKS	WEEK 6	WEEK 12		WEEK 13 – 14 (same animals)	
CODE	Administration CFE & basic chow	Faecal Collection	No	CODE	Randomized Food stimuli tests	No.
SNO - CFE 33	Snord116del - 33mg/kg		12	(2 -5 days between groups).		48
WT - CFE33	WT - 33mg/kg		12	2DG	a) Glucoprivic sig.	
SNO - CFE100	Snord116del - 100mg/kg		12	MA	b) Fatty acid sig.	
WT - CFE100	WT - 100mg/kg		12	SAL	c) saline	
SNO - PLAC	*Snord116del* - basic chow		12	SB	d) 5-HT2cR antagonist	
WT - PLAC	WT - basic chow		12	FAST	e) 50% food restriction	
TOTAL						48

Table 9. Presents the protocol for study two; numbers and delineated groups between the *Snord116del* (SNO) and (WT) mouse model. CFE – *Caralluma fimbriata*; 2DG - 2-deoxy-glucose; MA – beta-mercaptoacetate; SA – saline and SB – SB242804 (5-HT2c receptor antagonist).

5.4.6.2 Measurement procedures

Treatment measurements were taken over the eight-week chronic period and the treatment was administered over eleven weeks till completion of the experiments (SNO & WT x 2 doses CFE 33mg/100mg, n=48) against controls (PLAC, SNO & WT, n=24). A tared balance was used to weigh each treatment, which was then placed in each single animal. The dose weight was recorded daily.

To measure the daily food left over (FLO) a larger amount of food than would naturally be eaten was measured, offered and recorded as the amount given to the animal for each day. This FLO was collected at the same time period each day over a cyclic rotation through the groups. Therefore, each treatment group cycled from first to be recorded for FLO to sixth collection daily, over the full period of eight weeks. The measured full amount and FLO for each animal was recorded in the lab log book each day and at baseline, post experiment (after four hours) during the appetite signalling tests and on the fifth day after the four days of 50% reduction.

Fluid volumes were measured at baseline and post experiment, to determine the volume of water drunk by each animal from their dispenser during the appetite signalling tests. To achieve this the investigator utilized one non filled fluid bottle (dry) with a fixed rubber stopper for all the taring of fluid throughout all experiments. This bottle was kept aside in a cupboard just to tare the balance before each measurement. Basic chow pellets weighed for the 50% food restriction experiment were kept aside from the Barastoc custom mix as rations between 1.5 - 1.9g s. These chow pellets were sorted into weighed groups before the experimentation so they could be distributed at the necessary amount for each animal's half mean daily intake.

5.4.7 Statistical analysis

Statistical analyses for every experimental protocol within study two were performed using the IBM SPSS version 22.0 for Windows. Initial data was recorded in a laboratory log exercise book and transferred to an excel spread sheet, which was double checked at a different time line for any mistaken transfers of data. Statistical significance was defined at 95% confidence intervals.

5.4.7.1 Statistical analysis of appetite

Statistical analyses of appetite data were performed by collecting the sum of the FLO after free access to pre-measured chow during the dark cycle, over the full time period of eight weeks. The accumulative score for each animal was determined as mean □ SD. These accumulative appetite ingestion data were evaluated by SPSS as normally distributed, determined by Shapiro-

Wilk's and were evaluated for significance against all other strain/treatment scores using independent unpaired, *t*-tests and ANOVA for the groups' multivariate analyse. The differences between strain and treatments (2 x strain and 3x treatments groups) against all other strain/treatment scores: [(SNO (n=12) & WT (n=12) x 2 doses CFE 100mg &33mg/kg/d (n=48) & PLAC: SNO (n=12) & WT (n=12)]. These data are also documented for discussion (without statistical analysis) for any relationships between them, weight or hyperphagic drive.

5.4.7.2 Statistical analysis of bodyweight

Statistical analyses of body weight were determined by analysing the accumulative weight of each animal for all data over the eight-week period and determining the mean ±SD weight overall for each animal. The mean ±SD weight [(SNO (n=36) & WT (n=36)] were analysed against each other group between the strains and within the treatment groups using $P \leq 0.05$ as significant. As in appetite all data were normally distributed as determined by Shapiro-Wilk's. All *P* values were determined using independent unpaired, *t*-tests and ANOVA for the groups' multivariate analyse.

5.4.7.3 Statistical analysis of faecal gross energy

The data analysis for the weight of the samples and the HE of the single samples and group overall mean, were evaluated by using independent unpaired, *t*-tests and ANOVA for the groups. Comparisons of sample 1 and sample 2 were made for each animal - to make sure there were no remarkable variables. Once the parameters were balanced for each animal's samples, each full treatment group's samples (n=12; 24 samples) were analysed against each other treatment group and the results were expressed in mean ± SD, with $P = \leq 0.05$ considered significant. The interactions between the digestion, food eaten or the parameters of the treatment given, the dose or the animal's genotype in the results of the faecal collection, comparisons are considered in the discussion.

5.4.7.4 Statistical analysis of appetite signalling experiments

The complex appetite signally analysis began with checking the assumption of normalcy was met before assessing the two-way multi variant analysis of variance by a Two-way MANOVA. To achieve this the residual statistics were checked to determine if the output figures were within the bounds of the assumption of normality. Normalcy was defined by a few different statistical tests. Firstly, it was assumed that normality was met in sphericity of the groups as all groups were equally distributed with no missing values [SNO: n=36; WT: n=36: (100CFE/M: n=6 & F: n=6; 33CFE/M: n=6 & F: n=6; & PLAC/M: n=6 & F: n=6)]. Next the Box's Test of Equality Covariance Matrices Sig value was defined, set at a value of (P = > .005). The linear level relationship was determined through observation of a scattered dot plot and the matrix scatter mean line. Further images of multicollinearity were observed to determine if the scatter followed the same angle from lower left to top right.

From here the analysis was two-fold and complex, there were two dependent variables of strain (SNO and WT), three dependent variables for treatment (100CFE/kg/d, 33CFE/kg/d & PLAC) and four independent variables stimulating appetite variables (control-SAL, the appetite stimulants 2DG & MA & the antagonist SB 242084). The strain and treatment needed to be defined as the base whilst the stimulants altered the outcome. Similarly, the strain and treatment were capable of affecting the stimulants outcome, altering the mean and SD per group for both food and fluid intake due to each reagent. Therefore analysis utilized a Two–way MANOVA for significance, followed by a Repeated measure ANOVA for univariate measures. This determined the significance of the reagents against both strain and treatment as between group factors of variance. This was then followed by unpaired *t*-test for each reagent against saline as the control for within group factors of variance to answer the specific questions Section 5.4.6.1 . All results are tabled in Section 5.5, with mean and standard deviations at a value of $P \leq 0.05$ as significant. A visual representation of the later interactions is presented in graphs Figures 18, 19 and 20.

5.4.7.5 Statistical analysis of 50% fast in animals

In this experiment the dependent variables of treatment and strain were analysed for alterations of variance. This was accomplished by collecting the remaining food and water after 24 hrs, which was statistically analysed for the variance between groups [SNO: n=36; WT: n=36:

(100CFE/M: n=6 & F: n=6; 33CFE/M: n=6 & F: n=6; & PLAC/M: n=6 & F: n=6)] using an ANOVA.

5.5 Results

5.5.1 Results of the Eight-week chronic treatment study, in mice.

The results for appetite present a statistical analysis of the mean and standard deviation for each animal/group determined by collecting FLO from the pre-measured food, left for ad libitum feeding over 24 hours incorporating the dark cycle. The values presented in table 10 and in Appendix K, are over the eight-week treatment trial.

Table 10: Results for the Eight-Week Chronic Treatment Trial

Daily BC intake in (g)	Mean	SD (n=12)		Mean	SD (n=12)	P value
SNO 100CFE	3.32	± 0.67	WT 100CFE	3.59	± 0.73	0.005**
SNO 33CFE	3.36	± 0.67	WT 33CFE	3.36	± 0.66	0.10
SNO PLAC	3.40	± 0.71	WT PLAC	3.49	± 0.97	0.35
SNO 100CFE	3.49	± 0.20	WT 100CFE	3.59	± 0.73	0.29
SNO 33CFE	3.52	± 0.20	WT 33CFE	3.36	± 0.66	0.76
SNO PLAC	3.57	± 0.24	WT PLAC	3.49	± 0.97	0.34
Within strain t – test pairing: same animals (n=12) per grp						P value
SNO 100CFE & 33CFE						0.67
SNO 100CFE & PLAC						0.38
SNO 33CFE & PLAC						0.65
WT 100CFE & 33CFE						0.33
WT 100CFE & PLAC						0.34
WT 33CFE & PLAC						0.95

Table 10 Appetite unpaired *t*-tests over an eight-week period from baseline to the end of treatment demonstrating the mean and standard deviation (SD) of the daily basic chow (BC) ingested when treated with CFE – Caralluma fimbriata X 2 doses (100mg/kg/d & 33mg/kg) or PLAC - placebo of maltodextrin/cabbage leaf, in the Garvan Snord116del mouse model (SNO): (100CFE/M: n=6 & F: n=6; 33CFE/M: n=6 & F: n=6; & PLAC/M: n=6 & F:n=6)] against the C57BL/6 wild type (WT) strain (100CFE/M: n=6 & F: n=6; 33CFE/M: n=6 & F: n=6; & PLAC/M: n=6 & F:n=6)]. *T* – test scores also present within strain comparisons of treatment outcomes.

The results determine the differences in appetite due to CFE treatment - over the eight-week period – as minimal, with the only significance being between the 100CFE groups: - SNO100CFE: 3.32 ± 0.67, WT-100CFE: 3.59 ± 0.73, (P = 0.005). The SNO-100CFE ingested the lowest amount of food over each group and WT 100CFE ingested the highest amount of food.

In regards to the hyperphagia expected in the SNO animals, this was distinguishable when altering their food intake for the -5% body weight difference leading to no significant between the WT and SNO animals. Further, on occasion all the SNO animals ate more food than the WT strain (Section 5.6.6.3) but over the length of eight weeks there was a variety within range, where very large amounts were followed by very low amounts of food in the SNO animals which led to no significant hyperphagia. The largest amount of food eaten in one sitting (not counting the deprivation studies) was 6.5 grams in the SNO-PLAC group and on many occasion the SNO mice would eat up to 5 grams nightly. These values therefore sit within the overall data.

5.5.2 Study two body-weight

On the whole the SNO animals were 5% smaller in body weight. Even so there were no significant differences in the animal's weight gained over the eight weeks of treatment. The strongest differences are shown in table 11, in regards to the differences between strain values.

Table 11 Body Weight Results

Presents mean and standard deviation of body weight gain over the period of eight weeks during the chronic appetite tests in the Snord116del mouse model and WT control during CFE/PLAC treatment.

Treatment	BL in (g)		End in (g)		Total B/W gain over 8 wks in (g).	
(n=12) B/W/g	Mean	SD	Mean	SD	Mean	SD
SNO-100CFE	16.17	2.17	20.07	2.08	3.90	1.55
SNO-33CFE	16.34	2.00	20.23	1.81	3.88	1.22
SNO-PLAC	15.78	2.49	19.74	2.18	3.96	1.71
WT-100CFE	18.03	2.61	21.88	2.47	3.85	1.36
WT-33CFE	17.18	2.01	21.16	2.08	3.98	1.11
WT-PLAC	17.33	2.05	20.81	2.40	3.47	0.66

Within strain *t* – test pairing	BL *P* value	End *P* value	Total *P* value
SNO 100CFE & 33CFE	0.83	0.84	0.83
SNO 100CFE & PLAC	0.69	0.71	0.68
SNO 33CFE & PLAC	0.55	0.56	0.54
WT 100CFE & 33CFE	0.38	0.45	0.37
WT 100CFE & PLAC	0.47	0.29	0.31
W T 33CFE & PLAC	0.88	0.70	0.91
SNO 100CFE & WT 100CFE	0.07	0.06	0.93
SNO 33CFE & WT 33CFE	0.31	0.25	0.77
SNO PLAC & WT PLAC	0.11	0.26	0.41

Table 11. Unpaired *t* –tests, mean and standard deviation (SD) of body weight B/W/ in grams (g), over treatment with CFE – *Caralluma fimbriata X* 2 doses (100mg/kg/d & 33mg/kg PLAC - placebo of maltodextrin/cabbage leaf treatment group, in the Garvan *Snord116del* mouse model (SNO) : (100CFE/M: n=6 & F: n=6; 33CFE/M: n=6 & F: n=6; & PLAC/M: n=6 & F:n=6)] against the C57BL/6 wild type (WT) strain (100CFE/M: n=6 & F: n=6; 33CFE/M: n=6 & F: n=6; & PLAC/M: n=6 & F:n=6)]. The table results explore unpaired *t*-tests of weight at baseline (BL), at the end (END) of 8 weeks (wks) and as an accumulative mean and SD for body weight (BW) gained over 8-wks, also exploring within strain comparison and against other strain's same treatment group.

The unpaired *t* – tests Table 11presents the results of the mouse group weights showing mean and SD scores for the 'within strain' measures of treatment in the *Snord116del* mice and WT mice in *t* -tests against each treatment group and between strain, against the opposite strain's treatment values SNO: n=36; WT: n=36: (100CFE/kg/d, n=12; 33CFE/kg/d n=12; & PLAC n =12). These values are presented in grams (g), as measures for baseline, end of an eight-week treatment cycle, and as mean and SD of the accumulative values for the daily weight scores (to define the weight gained for each group).

The scores present similarity at baseline before treatment with no significant difference between strains with the lowest group being the 100CFE. These weights changed with a little weight loss due to single caging. At Randomization all animal groups were similar. The weight was gained quickly and between strains there were significant differences over time with the lowest weight group once again being the SNO-100CFE at completion of the eight-weeks.

5.5.3 Food appetite, stimulant tests

The multi variant analysis of variance was very complex as there was more than one independent and dependent variable. Therefore, it was important to explore the fixed factor *t*ests for specific interactive variables after the Two-way MANOVA post hoc tests.

The following results utilize the same animals as the earlier chronic test and show the values at the end of the four hour tests after the earlier i.p. injections of saline, 2DG, MA and the 5-HT2c receptor antagonist: SB 242804. The results determined some unexpected levels in hunger activity and thirst. Firstly, these results demonstrate differences in appetite signalling tests during pairwise comparisons of hunger in the mouse models strains - where the animals had been ingesting CFE (33mg or 100mg/kg/d) or PLAC at between 10-12 weeks' treatment. Then overall, the differences observed for food intake were most strongly demonstrated between the two strains, due to 2DG: - SNO-100CFE 0.78 ± 0.37g; WT-100CFE 1.19± 0.18g, (P = 0.002), SNO-33CFE 0.98 ± 0.29g; WT-33CFE 1.36± 0.30g, (P = 0.002), SNO-PLAC 1.01 ± 0.26g; WT-PLAC 1.56± 0.67g, (P = 0.01) (Appendix I. unpaired *t*-tests, tables 29). The most powerful appetite stimulant in all the animals (over both strains and all treatment groups was the 5-HT2c receptor antagonist (Section 5.5.4). This suggests a blocking of serotonin capabilities from the suggested SSRI component to CFE and it also includes the placebo, which suggests normal serotonin levels were also blocked.

The results for the four-hour tests (Tables 12 & Figure 13), show the values for the stimulants compared to the control SAL to determine any significant difference. In regards to the SAL tests, the total SNO animals ate more food over the four-hours; - SNO 1.39 ± 0.32g; WT 1.14 ± 0.40g, (P = <0.001). On the same animal numbers (n=36) per strain, the outcomes were also significant, though opposite in effect during 2DG i.p. administration: SNO 0.92 ± 0.31g; WT

1.36 ± 0.45g, (P = <0.001). This alteration is discussed in the behavioural characteristics Section 5.6.6. In regards to 2DG administration when considering the treatment effects (SNO paired with WT n=24), there was a significant difference during 2DG administration SNO100CFE/kg/d 0.77 ± 0.37g paired with WT-100CFE/kg/d 1.19 ± 0.18g, to SNO-PLAC 100 ± 0.25g paired with WT PLAC, 155 ± 0.55g, (P = 0.02). When pairing the SNO and WT 33CFE/kg/d whilst being administered 2DG, the accumulative appetite scores were not significant (NS).

In comparing the significant differences within the stimulation/or control, between each specific group (n=12), there were some results that determined appetite activity due to CFE. SNO animals' food consumed on SAL between CFE doses: - SNO-100CFE 1.21 ± 0.27 1.47; SNO33CFE 0.23 ± 0.03 (P = 0.03) (Appendix I. *t*-test SAL) and food consumed between strains on 2DG.

This was very unusual as glucose deprivation through 2DG administration would be expected to stimulate feeding. In the SNO animals' 2DG resulted in the lowest intake of chow SAL: - SNO-100CFE 1.21g ± 0.27g, 2DG: - SNO-100CFE 0.78g ± 0.37g, (P = <0.001), SAL: SNO33CFE 1.47g ± 0.23g, 2DG: - SNO-33CFE 0.98g ± 0.29g, (P = <0.001) and SNO PLAC 1.46g ± 0.41g, 2DG: SNO 1.01g ± 0.26g, (P = 0.003). As expected i.p injections of 2DG stimulated feeding in the WT animals, SAL: - WT-100CFE 0.81g ± 0.27g; 2DG: - WT-100CFE 1.19g ± 0.18g, (P = <0.001), SAL: - WT-33CFE 0.94g ± 0.30g; 2DG: - WT-33CFE 1.36g ± 0.30g, (P = 0.002) and WT-PLAC 0.91g ± 0.39g; 2DG: WT 1.56g ± 0.67g, (P = 0.008). The different levels of feeding were once again less in the 100CFE group.

In the case of food intake during MA administration against the control, the data was also significant in the SNO animals against with minimal change in appetite for the WT animals, SAL: - SNO-100CFE 1.21g ± 0.27g; MA: - SNO-100CFE 0.92g ± 0.34g, (P = 0.02), SAL: SNO-33CFE 1.47g ± 0.23g; MA: - SNO-33CFE 1.05g ± 0.37g, (P = 0.003) and SNO-PLAC 1.46 ± 0.41, MA: SNO 0.94g ± 0.31g, (P = 0.003). In this case there was not such a clear

variation due to CFE. In the WT animals the differences were minimal and not significant. Behaviour in water intake during this treatment was very significant (Table 13).

5.5.4 Food appetite: 5-HT2cR antagonist SB 242084

In experiment four the study was ultimately looking to identify the role of the 5-HT2c receptor in CFE induced reductions of appetite. The 5-HT2cR antagonist; SB 242084 (Kennett et al., 1997) known to block the capacity for serotonin to interact with this receptor, was expected to cause the animals to become more hungry.

Under the hypothesis that CFE interacts through this receptor, SB 242804 correctly enhanced feeding for all groups. The results for food intake were not significantly altered in the SNO groups including the PLAC group, which confirms the blocking of normal serotonin. Significance was seen in the WT animals against SAL: - WT-100CFE 0.81 ± 0.27; SB 242804 WT-100CFE 1.61g ± 0.61g, (*P* = <0.001), SAL, WT-33CFE 0.94 ± 0.30; SB242804 WT33CFE 1.79g ± 0.70g, (*P* = <0.001) and in the PLAC group small trend (NS). Also within the four-hour period over all groups the antagonist strongly increased intake of chow against SAL (*P* = <0.001), as well as against the other stimulants 2DG, 1.14 ± 0.44g, (P = <0.001) and MA, 0.98 ± 0.33g, (P = <0.001). This once again confirms the SSRI qualities of CFE.

Table 12 Appetite Stimulation Study Two

Food: appetite signalling tests showing pairwise comparisons of hunger with significance set as Pillai's trace Sig value, ($P = \leq .05$) for food over 4 hrs.

Reagent	Strain	Treatment	P value
SAL	SNO vs WT	All	<0.001**
2DG	SNO vs WT	All	<0.001**
MA	SNO vs WT	All	0.89
SB 242804	SNO vs WT	All	0.89
SAL	SNO & WT	100CFE vs 33CFE	0.14
		100CFE vs PLAC	0.21
		33CFE vs PLAC	0.97
2DG	SNO & WT	100CFE vs 33CFE	0.22
		100CFE vs PLAC	0.02*
		33CFE vs PLAC	0.57
MA	SNO & WT	100CFE vs 33CFE	0.94
		100CFE vs PLAC	1.00
		33CFE vs PLAC	0.90
SB 242804	SNO & WT	100CFE vs 33CFE	0.59
		100CFE vs PLAC	0.67
		33CFE vs PLAC	0.17
SAL vs 2DG	SNO & WT	All	1.00
SAL vs MA			0.03*
SAL vs SB 242804			<0.001**

Table 12. Four-hour appetite signalling test pairwise comparisons of hunger in the mouse models, Garvan Snord116del (SNO) and C57BL/6 wild type (WT) strains [SNO: n=36; WT: n=36: (100CFE/M: n=6 & F: n=6; 33CFE/M: n=6 & F: n=6; & PLAC/M: n=6 & F: n=6)]. All animals were ingesting chronic treatment CFE: - Caralluma fimbriata extract, one of two doses 100mg/kg/d or 33mg/kg/d or PLAC: – placebo of maltodextrin/cabbage leaf. The table presents mean and SD: - standard deviation, comparisons of food ingested in grams (g), after 4 hrs due to the appetite signalling reagents: SAL: - saline control, 2DG: - 2-deoxy-glucose, MA: - beta –

Table 13 Comparisons of Stimulants

Food and water appetite level saline comparisons after i.p. injections of stimulants - 2DG, MA & the 5HT2c receptor antagonist SB 242084 in Snord116del and C57BL/6 wild type mouse model, utilizing a unpaired t-test of in comparison to saline											
Comparisons to SAL	SAL		2DG			MA			SB 242084		
	Mean	SD	Mean	SD	P value	Mean	SD	P value	Mean	SD	P value
FOOD SNORD116 (g)											
100CFE (n=12)	1.21	0.27	0.78	0.37	<0.001**	1.00	0.23	0.02*	1.46	0.38	0.11
33CFE (n=12)	1.47	0.23	0.98	0.29	<0.001**	1.05	0.37	0.003**	1.60	0.64	0.51
PLAC (n=12)	1.46	0.41	1.01	0.26	0.003**	0.94	0.31	0.003**	1.55	0.47	0.63
FLUID SNORD116 (ml)											
100CFE (n=12)	6.24	1.48	6.54	3.02	0.8	2.62	1.64	<0.001**	5.84	1.74	0.6
33CFE (n=12)	7.24	2.28	6.16	3.91	0.41	1.80	1.72	<0.001**	6.29	2.77	0.36
PLAC (n=12)	5.84	0.66	4.85	4.97	0.51	2.88	2.56	0.002**	3.84	0.92	<0.001**
FOOD WT (g)											
100CFE (n=12)	0.81	0.27	1.19	0.18	<0.001**	0.78	0.37	0.14	1.61	0.61	<0.001**
33CFE (n=12)	0.94	0.30	1.36	0.30	0.002**	0.98	0.29	0.55	1.79	0.7	<0.001**
PLAC (n=12)	0.91	0.39	1.56	0.67	0.008**	1.01	0.26	0.47	1.25	0.44	0.06
FLUID WT (ml)											
100CFE (n=12)	3.81	1.48	4.10	1.35	0.54	5.15	2.47	0.12	6.69	1.47	<0.001**
33CFE (n=12)	4.15	1.81	4.47	1.46	0.64	5.87	1.61	0.01*	8.37	3.42	0.001**
PLAC (n=12)	5.78	1.48	5.11	2.63	0.44	5.16	1.27	0.28	4.76	1.66	0.12

Table 13 presents unpaired t –tests with the mean and standard deviation (SD) of food in grams (g) and fluid in millilitres (ml). Food and fluid were ingested over 4 hours from baseline, in the Garvan Snord116del mouse model (SNO) and C57BL/6 wild type (WT) strain, after receiving one of four intraperitoneal injections of a stimulant or control. These include saline (SAL), 2-deoxy-glucose (2DG), beta – mercaptoacetate (MA) or the 5-HT2c antagonist SB242804. The groups all followed the same protocol randomized on different days and the results were categorized by within strain factors of treatment for the extract of Caralluma fimbriata (CFE) x 2 doses (100mg/kg/d & 33mg/kg) & the placebo of maltodextrin/cabbage leaf (PLAC). Table shows unpaired t-test, mean and standard deviations comparing the control SAL against (vs) 2DG, MA & SB 242804 with a P value of $P = \leq 0.05$ as significant.

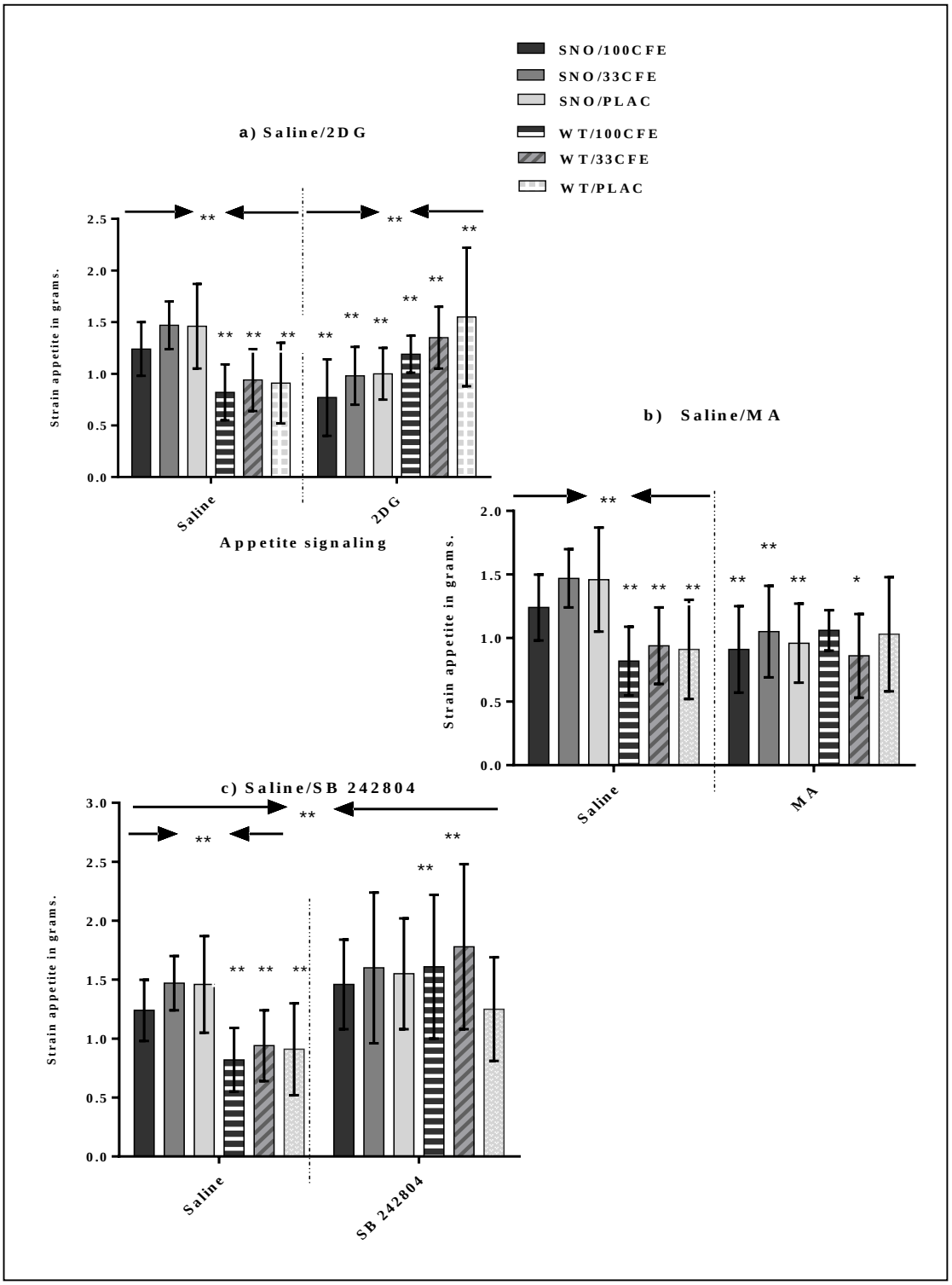


Figure 18 Appetite Stimulants Histograms

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Figure 18 Appetite Stimulants Histograms

Figure 18 graphs depict the univariate between-subject results for appetite signalling tests the saline comparisons to mean and SD: - standard deviation, comparisons of food ingested in grams, after 4 hrs due to the appetite signalling reagents: SAL: - saline control, 2DG: - 2-deoxy-glucose, MA: - beta – mercaptoacetate or the 5-HT2c antagonist SB242804. The results present pairwise comparisons for food ingested over four hours, with significance set as Pillai's trace Sig value, $P = \leq .05$, plus *t*-tests between three chronic treatment groups saline vs. appetite signalling. The animal models were the *Snord116del* (SNO) and wild type (WT) strains [SNO: n=36; WT: n=36: (100CFE/M: n=6 & F: n=6; 33CFE/M: n=6 & F: n=6; & PLAC/M: n=6 & F: n=6)]. All animals were ingesting a chronic treatment of either CFE: - *Caralluma fimbriata* extract, at one of two doses 100mg/kg/d or 33mg/kg/d or PLAC: – placebo of maltodextrin/cabbage leaf. The results of the Post Hoc *t*-tests, for the amount of chow and water consumed, after single i.p. stimulant tests and SB242804 are presented in Appendix K.

5.5.5 Water appetite, stimulant tests

The tests for thirst in the SNO animals determined some unexpected activity and results during administration of SAL, 2DG, MA and the 5-HT2c antagonist SB242804. Unusually during 2DG and MA administration the results between strains were significantly different (P = <0.001) due to opposite effects in the two strains. The stimulants and antagonist also determined opposite water appetite to food appetite in the SNO mice which was very unusual. The mean and SD are presented in Appendix Jb Table 30 & Figure19.

In regards to the four-hour values for water consumption between strains (n=36) the results are as follows: SAL: - SNO 5.32 ± 2.19ml; WT 6.60 ± 2.73ml, (P = 0.01), 2DG: - SNO 6.44 ± 2.22ml; WT 4.58 ± 1.78ml, (P = <0.001), MA:- SNO 2.44 ± 2.01ml; WT 5.48 ± 1.86ml, (P = <0.001). In regards to treatment (paired strain n=24), SAL: - SNO-100CFE 5.84 ± 1.74ml & WT-100CFE 6.69 ± 1.48ml, compared to SNO-PLAC 384 ± 0.92ml & WT-PLAC 4.76 ± 1.66ml, (P = 0.008), SNO-33CFE 6.29 ± 2.78ml & WT-33CFE 8.36 ± 3.42ml, compared to SNO-PLAC 384 ± 0.92ml & WT-PLAC 4.76 ± 1.66ml, (P = <0.001). These results suggest a misleading effect as there was an opposite effect between strains. The results between treatments are important to view within the *t* – test (Appendix I.b, table). In comparisons between the stimulants (n=72) there were significant levels of water consumed for SAL, 2DG and SB 242804 when compared to MA which was very low in the SNO animals, SAL 5.96 ± 2.54ml against MA 3.96 ± 2.46ml, (P = <0.001). This drop in water consumption during MA administration continued in the SNO groups. All mean and SD values are presented in Appendix J. In the WT animals there was significant difference in water intake during MA against the control SAL: WT-33CFE 4.15ml ± 1.81ml; MA WT-33CFE 4.87ml ± 1.61ml, (P = 0.02) and against the SB242804, SAL: WT-100CFE 3.18ml ± 1.48ml; SB 242804 6.69ml ± 1.47ml, (P = <0.001) and WT-PLAC SAL 5.78 ± 1.48 against SB 242804: 8.37 ± 0.42, (P = 0.001). Table 13 presents the results of the univariate test scores for water intake and comparisons are represented by graphs in Figure 19.

Importantly the SNO groups increased hunger during the 5-HT2c antagonist SB 242804 and this also increased thirst except in the SNO-PLAC group, SAL: 5.84ml ± 1.49ml; SB 242804 3.84ml ± 0.92ml, (P = <0.001). Due to this it is clear that CFE interacts with thirst as the raised thirst was only seen on the animals that had been ingesting CFE.

Table 14 Between Treatment and Strain Effects of Appetite Stimulation

Between-subjects effects for water appetite signalling tests showing pairwise comparisons with significance set as Pillai's trace Sig value of $P = \leq .05$ over 4 hrs.

Reagent	Strain	Treatment	P value
SAL	SNO vs WT	All	0.01*
2DG	SNO vs WT	All	<0.001**
MA	SNO vs WT	All	<0.001**
SB 242804	SNO vs WT	All	0.93
SAL	SNO & WT	100CFE vs 33CFE	0.28
		100CFE vs PLAC	0.008**
		33CFE vs PLAC	<0.001**
2DG	SNO & WT	100CFE vs 33CFE	0.69
		100CFE vs PLAC	0.47
		33CFE vs PLAC	1.00
MA	SNO & WT	100CFE vs 33CFE	1.00
		100CFE vs PLAC	1.00
		33CFE vs PLAC	1.00
SB 242804	SNO & WT	100CFE vs 33CFE	1.00
		100CFE vs PLAC	1.00
		33CFE vs PLAC	1.00
SAL vs 2DG	SNO & WT	All	0.93 <0.001**
SAL vs MA			
SAL vs SB 242804			0.50

Table 14**.** Four-hour appetite signalling test, pairwise comparisons of thirst in the mouse models, Garvan *Snord116del* (SNO) and C57BL/6 wild type (WT) strains [SNO: n=36; WT: n=36: (100CFE/M: n=6 & F: n=6; 33CFE/M: n=6 & F: n=6; & PLAC/M: n=6 & F: n=6)]. All animals were ingesting chronic treatment CFE: - *Caralluma fimbriata* extract, one of two doses 100mg/kg/d or 33mg/kg/d or PLAC: – placebo of maltodextrin/cabbage leaf. The table presents mean and SD: - standard deviation, comparisons of water ingested in millilitres (mls), after 4 hrs due to the appetite signalling reagents: SAL: - saline control, 2DG: - 2-deoxy-glucose, MA: - beta – mercaptoacetate or the 5-HT2c antagonist SB242804.

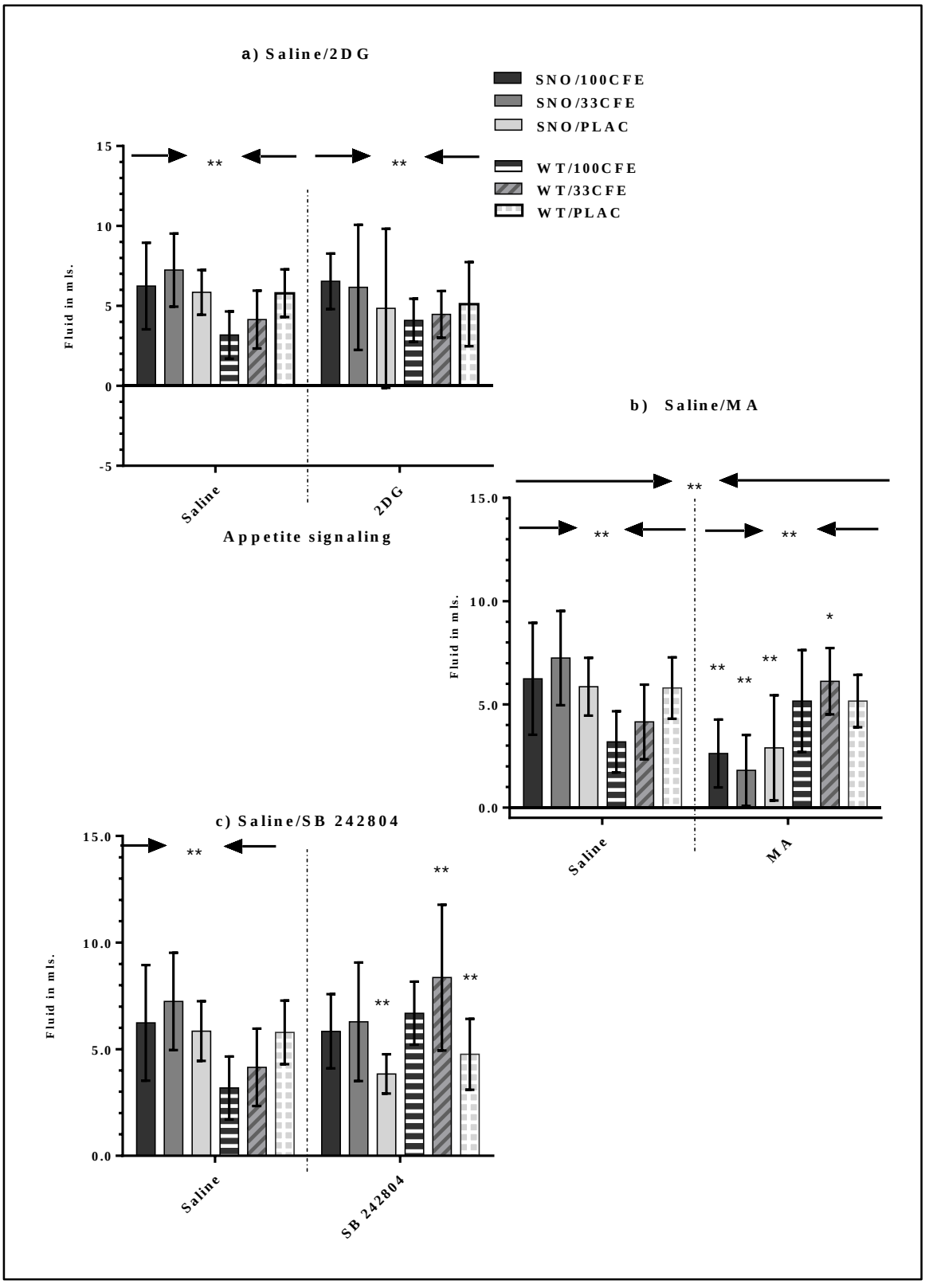

Figure 19 Water Appetite during Stimulation Study

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Figure 19 Water Appetite during Stimulation Study

Figure 19 presents univariate between-subject results for water appetite signalling tests over four hours, showing pairwise comparisons for thirst with significance set as Pillai's trace Sig value, ($P = \leq .05$). The animal models were the *Snord116del* (SNO) and wild type (WT) strains [SNO: n=36; WT: n=36: (100CFE/M: n=6 & F: n=6; 33CFE/M: n=6 & F: n=6; & PLAC/M: n=6 & F: n=6)]. All animals were ingesting a chronic treatment CFE: - *Caralluma fimbriata* extract, one of two doses 100mg/kg/d or 33mg/kg/d or PLAC: – placebo of maltodextrin/cabbage leaf. The table presents mean and SD: - standard deviation, comparisons of fluid (water) ingested in millilitres (mls), after 4 hrs due to the appetite signalling reagents: SAL: - saline control, 2DG: - 2-deoxy-glucose, MA: - beta – mercaptoacetate or the 5-HT2c antagonist SB242804.

5.5.6 Fifty percent deprivation experiments

Further study determined the interaction between the treatment, genetic deletion and the intake of food and fluid after a period of four days during 50% food deprivation (Figure 16). The average daily intake over 24 hours for each animal was determined during the eight-week chronic administration of treatments CFE at both doses (100mg/kg/d & 33mg/kg) and PLAC. This was then halved and given daily at the time of treatment. Amounts given ranged from 1.5 – 1.9 grams. Comparisons of food after deprivation showed no significance. Comparisons of water intake after the four days demonstrated significant differences between the two strain's treatment groups: SNO-100CFE 9.07 ± 1.48ml; WT-100CFE 11.80 ± 2.92ml, ($P = 0.03$), SNO-33CFE 8.22 ± 2.81ml, WT-33CFE 12.66 ± 1.62ml, ($P = <0.001$) and SNO-PLAC 8.09 ± 2.08ml; WT-PLAC 11.52 ± 2.08ml, ($P = <0.001$).

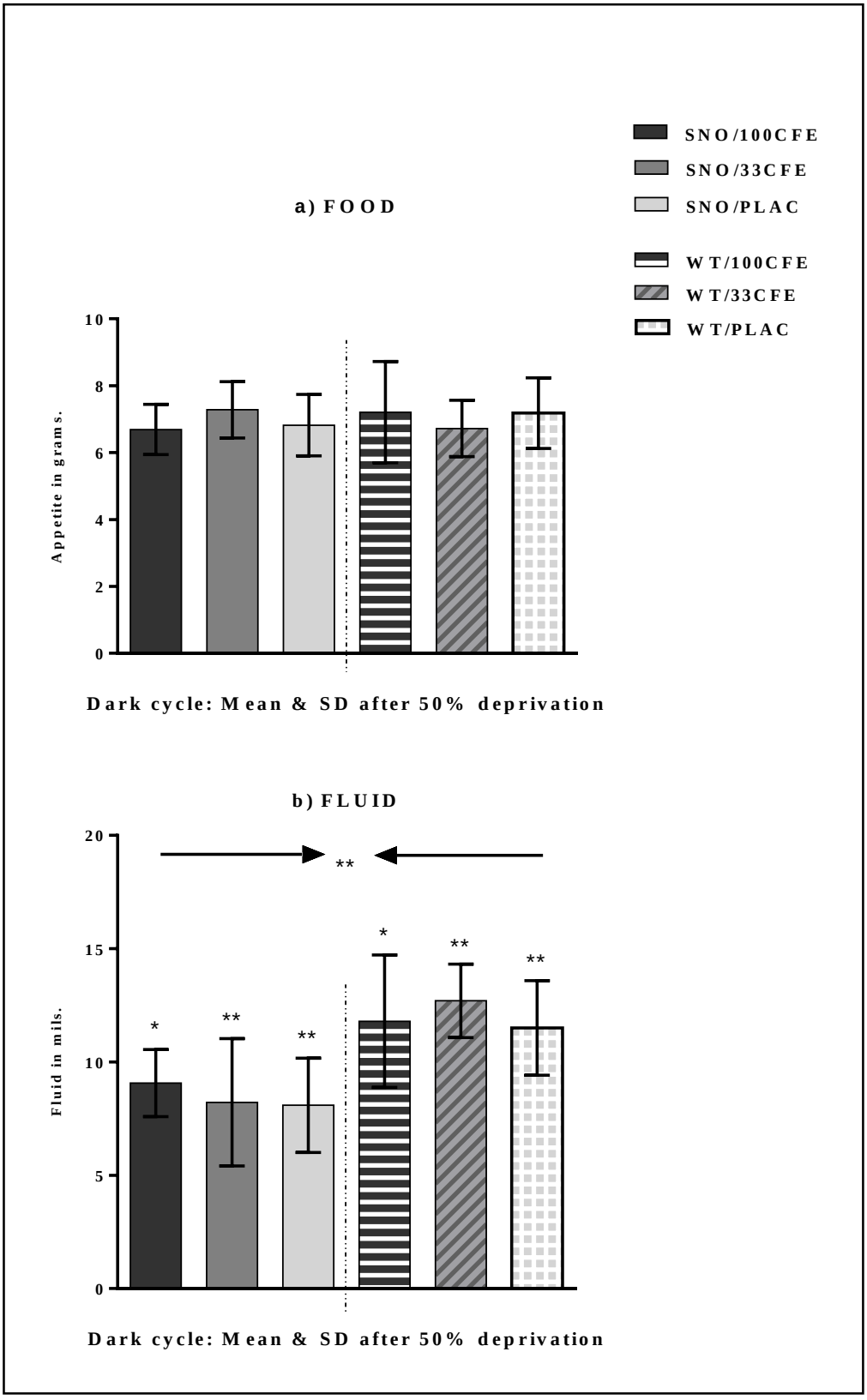

Figure 20 Food and Water after Four Days 50% Deprivation
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Figure 20 Food and Water after Four Days 50% Deprivation

Figure 20 presents unpaired *t* –tests, mean and SD: standard deviation, of food and fluid ingested after four days of 50% deprivation from baseline, in the SNO: Garvan *Snord116del* mouse model compared to C57BL/6, WT: wild type strain. Mouse models received 50% of their basic chow (set from average dark cycle feed) and water ad libitum, during chronic administration of treatments CFE: extract of *Caralluma* fimbriata x 2 doses (100mg/kg/d & 33mg/kg) against PLAC: placebo of maltodextrin/cabbage leaf.

5.5.7 Faecal discharge

The objective of collecting faecal discharge was twofold. One to determine if the *Snord116del* animals were able to digest and absorb the chow and two, to determine if any dietary efficiency established by the experiments in CFE was in anyway attributed to the faecal output. Dietary intake which was eliminated in the excreta was observed to increase the HE by CAL2K in only the WT animals on 100CFE as follows: - WT-100CFE 61.75 ± 20.82mj, SNO-100CFE 26.63 ± 2.32mj, (P = <0.001) and against all other treatments in both strains (P = <0.001). The total treatment results (n=72) also demonstrated a significant difference between 100CFE 44.19 ± 23.06mj, (P = <0.001), the other dose 33CFE 27.11 ± 5.28mj or PLAC 25.87 ± 2.99mj. Post hoc analysis confirmed these differences were due to the WT animals (Table 15).

Table 15 Results for Faecal Sample Testing in Animals

Combined average faecal HE from Snord116del and WT control group faeces (n=12) at two doses of CFE or PLAC maltodextrin/cabbage leaf.

Strain	SNO						WT								
Parameters All mice	100CFE		33CFE		PLAC		100CFE		33CFE		PLAC		TOTAL		P Value.
(mj)	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	
Female (n=6)	26.20	1.26	29.65	5.02	25.79	2.78	60.70	15.61**	25.21	8.97	24.68	1.31			<0.001**
Male (n=6)	27.05	3.12	27.40	1.59	25.25	1.54	62.79	26.60**	26.17	2.61	27.76	4.77			<0.001**
Total (n=12)	26.63	2.32	28.53	3.74	25.52	2.16	61.75	20.82**	25.69	6.31	26.22	3.71			<0.001**
Total treatment 100CFE (n=72)													44.19	23.06**	<0.001**
33CFE (n=72)													27.11	5.28	<0.001**
PLAC (n=72)													25.87	2.99	<0.001**

Table 15 presents mean and SD: - standard deviation of pairwise comparisons for faecal heat energy (HE) in millijoules (mj) from a Calorimetric measurement of a powdered sample of three day's collection for each animal. Mouse models, Garvan *Snord116del* (SNO) and C57BL/6 wild type (WT) strains [SNO: n=36; WT: n=36: (100CFE/M: n=6 & F: n=6; 33CFE/M: n=6 & F: n=6; & PLAC/M: n=6 & F: n=6)]. All animals were ingesting chronic treatment CFE: - *Caralluma fimbriata* extract, one of two doses 100CFE: -100mg/kg/d or 33CFE: - 33mg/kg/d or PLAC: – placebo of maltodextrin/cabbage leaf.

5.6 Discussion

Though the mean results of appetite activity in either the *Snord116del* or WT control mice - over the eight-weeks chronic administration of CFE/PLAC - do not demonstrate a significant attenuation of appetite due to CFE, the appetite stimulant interactions of food and fluid, plus the day to day behavioural characteristics of the animal's hunger (Appendix I), did determine some interesting outcomes. Certainly there was a trend in reduction over the two doses in the SNO-100CFE animals but the increase in appetite was unexpected in the WT-100CFE animals. This difference in how the highest dose interacted with the strains could initiate many plausible hypotheses though clearly this was obviously in part, due to malabsorption, determined by the faecal experiments. The hyperactive behaviour (Appendix I.a/) may have had some influence, plus the difference in absorption of nutrients and perhaps even neuronal signalling of leptin (Clement, 2000) due to difference in adiposity related to the strains (Qi et al., 2016). This adiposity difference was noted during the next chapter's perfusion protocol. The organs in the SNO animals were particularly free of any fat deposits. Perhaps this was a reason the SNO-100CFE group of mice were only minimally able to maintain their stamina during the 50% deprivation. Unfortunately, fat deposits were not measured during the study.

In regards to the behavioural differences between the strains and questions related to how the SnoRNA deletion interacts with both CFE and appetite stimulation, the most interesting aspects were the opposite outcomes food appetite during 2DG administration and water appetite during MA. The other interesting behavioural aspects were the unusual spinning in the SNO animals during the 50% deprivation trial (Appendix I h/) and the cyclical daily appetite (Appendix I c/) Certainly the hyperphagia expected in the SNO animals, was distinguishable when altering their food intake for the -5% bodyweight adjustment between strains. Importantly also in relation to behavioural observations, energy levels were exaggerated when the animals were ingesting CFE100mg/k/d. Those on 100CFE seemed to be hyperactive, which may naturally determine a need for more food. It may be important to look at the amount of calories consumed and burnt through activity whilst on CFE in further research. This could also include size and weight, relative to calories. In regards to size, as expected the SNO strain were smaller at baseline (Qi et al., 2016) though their growth was not stunted and they were not significantly different in weight within the study. Overall the 5% difference in size would have meant only a little less energy was exhausted

during the hyperactivity. Interestingly it was hypothesized that CFE would alter the weight of the animals, which was not the case.

Compared to other hyperphagia related trials the weighing of the FLO daily enhanced the characterizing of how the hyperphagia was exhibited in the SNO animals. Daily results showed hyperphagic activity that was cyclical in nature - especially when adjusting their total amount eaten per day for the difference in weight against the WT controls. The cycles in appetite followed a pattern where an exaggeratedly high amount of food on one day, was followed by a couple of lower feeding days. For example, a 5g amount of food eaten over a 24-hour cycle was followed by two 2g days and then once again back to a 5g day. This was only in the SNO mice as the WT were more likely to have three 3g days in a row. Other research into the hyperphagia seems to take short term observations which may have missed this fact.

During 2DG the reduction in appetite was experienced with a strong BALD reaction where the stimulant obviously compromised glucose signalling uptake. These reactions during the signalling experiments need further study, mainly to define what signal is altering the need for food and water intake.

The appetite signalling experiments specifically focussed mechanisms of fuel sensing - glucose sensing, fatty acid oxidation and the mechanisms associated with 5-HT2c appetite activity within the CNS (Schellekens et al., 2015) (Section 4.3). Naturally the body effectively maintains glucose levels in both humans and animals by balancing the amount of energy utilized and the amount of food/fuel consumed (Oh et al., 2015, Sherwood, 2015). Indicators of alterations within this balance are lowering of the heart rate, slowing of the metabolism, decreased movement, shivering and altered thermoregulation – as seen in the BALD posture 9Appendix I). In life, even for mice, starving is not an option, when fuel is inaccessible, the set point of the metabolism will be readjusted to withstand a lower supply of fuel. As the study utilized chronic alterations of metabolic fuel (within short periods), alterations of the balanced set-point wasn't possible, therefore the animals would naturally be driven to feed, or in the case of the 2DG injections in the SNO animals on CFE, this culminated in a BALD posture. Therefore, the behavioural somnolence in the SNO animals may have been a conservation of energy or something like a diabetic episode. Further looking at the behavioural characteristics in the mice, these unusual behaviours of stillness or at times the opposite - vigorous speed – may indicate alterations in oxygen levels or ATP availability. The WT mice were more likely to go back to sleep when disturbed compared to the SNO mice which may indicate a different

moderation of ATP between strains. ATP or food-derived energy comes from a chemical reaction. Acetyl-CoA is a metabolic molecule where its main function is conveying the CoA - in an acetyl group - to the citric acid cycle to be oxidized in the cells mitochondrion for the production of ATP. CoA consists of a β-mercaptoethylamine group (related to the MA experiments) and the acetyl-CoA structure is produced by glycolysis and beta oxidation of fatty acids. The citric acid cycle oxidizes the acetyl group to carbon dioxide and water, whist energy is captured as ATP relative to the amount of acetyl that enters the cycle. An inhibitory action of CFE may have reduced the action of ATP reducing the acetyl-CoA pool, which is required for fatty acid biosynthesis (Foster, 2012) This could also cause a difference in lipid biosynthesis (Li et al., 2005). This reduction can also cause a suppression of body fat accumulation.

This process is of course different in the liver, in regards to the lack of glucose and the fasting experiments where under these circumstances gluconeogenesis in the liver creates glucose to be transferred to the brain. Steps convert oxaloacetate into glucose which is released into the blood as glucose. Also in the liver, when oxaloacetate is unavailable as will have been the case sometime into the fasting regime, two acetyl-CoA molecules follow a process to form the breakdown product, acetone or ketones which are released into the blood. The mitochondria then converts them to acetyl-CoA for fuel through the citric acid cycles which may be utilized by cells and the CNS. This may have been part of the WT interaction with CFE leading to an altered faecal HE. In the animals within the study it was obvious that the availability of acetylCoA molecules was at times restricted. Or in in the 50% deprivation, the cognitive state may have been an adjustment in blood flow, associated with lower oxygen levels (Li et al., 2005). The SNO mice were certainly demonstrating signs of some sort of reactive, repetitive drive with the spinning on the top of their cages.

Overall the animals' alterations in appetite and water intake, were closely linked with inactivity or hyperactivity due to the treatments given. This leads to a hypothesis that the results are linked as suggested above, to metabolic compensation. It seems important to address the fact that the SNO animals ate more than the WT during the four-hour testing of appetite due to the experimental control SAL.

During MA administration peripheral metabolic behaviour was noticed as typical sleepiness during the light cycle. This could have been natural or due to the blocking of β-oxidation of fatty acids through blocking mitochondrial acetyl-CoA dehydrogenesis. In the SNO animals

woken for MA injections there seemed less activity and perhaps a systemic dysfunction i.e. mitochondrial, or neurological (Yazdi et al., 2013) which determines the low water intake (Grossman and Grossman, 1963) Also sometimes the outcomes were more non-descript i.e. a little feeding here and there with no singular pattern which is hard to define. Perhaps too some of the interactions in the SNO animals - especially in regards to the blocking fatty acid – are related to the genetic deletion altering appetite pathways i.e. disrupted vagel afferents may increase hyperphagic patterning in these animals. This could mean that the SNO animals are used the experience of lower fatty acids availability in regards to metabolic fuel (Li et al., 2016). In the SNO mice on CFE another hypothesis may be that the reduction strongest reduction in thirst due to CFE, may have some relationship to fatigue (due to hyperactivity) or perhaps a lower citric acid cycle due to the limited acetyl-CoA dehydrogenesis shifting salt/electrolyte balance (Denton, 2006, McKinley et al., 2004). Lastly water appetite may become a response to dehydration lead by the osmolarity of electrolyte fluid-mineral balance. It may be that the brain contains serotonergic neurons which project information forward toward the forebrain for the regulation of the electrolyte composition. MA may have interacted with the sensory information altering or correcting electrolyte fluid–mineral balance. This adjusts many processes in the body including vasopressinergic (regulating blood pressure), oxytocenergic (involving renal sodium absorption) and serotonergic modulation of the CNS (McKinley et al., 2004).

Though this thesis hypothesizes that CFE interacts in some way with ATP and adiposity, it was exceptional to have clearly determined a mechanism of action for CFE. Under the hypothesis that CFE interacts through a serotonin signalling mechanism, the appetite signalling studies did confirm CFE to have SSRI properties. The 5-HT2c receptor antagonist, SB 242804, correctly enhanced feeding against the control SAL within all the animals. This was only significant in the WT-CFE 100/33mg groups and the SNO-100CFE group. These results were also significant in all the animals against the other appetite signalling reagents. These high appetite levels were due to the blocking of the 5-HT2c receptor. As discussed in section this receptor's downstream pathway is defined as an anorexigenic one, so therefore the animals exhibited an exaggerated hunger due to differences in hypothalamic regulation of serotonin (Ding et al., 2008, Ding et al., 2005, Di Matteo et al., 2001, Clifton et al., 2000, Bortolin-Cavaillé and Cavaillé).

Some of the complexities of the altered appetite have been characterized in this thesis. The significant differences between the strains confirmed that the *Snord116del* mouse model was a

viable choice in this complex study of a treatment for the hyperphagia in PWS. The next chapter investigates more mechanistically the appetite alterations determined during 2DG administration and determines if further study of the investigates in water consumption alterations due to MA is warranted as further study.

5.6.1 Limitations

It is inevitable that there will be limitations to the resources and time within a PhD candidature. It was with regret that the time frame limited enlisting more animal studies. Unfortunately, too there were limitations statistically in regards to water values, due to faulty equipment. The water intake tests were not as stringent as was hoped. Though all care was taken to stop water dripping into the houses, there were some cases of leaking equipment over the four-hour studies. (12/288 tests). In these cases - where wet ground cover was seen - to stop over-estimation of water, the scores were taken from the mean of the same treatment group. This determined there were no missing values. To help these tests become more stringent, the multivariate test Pillia's Trace was defined for significance instead of Wilks Lambda.

In regards to the administration of the treatment. It may be typical to administer a treatment through a gavage technique. The researchers believed this technique could have become technically challenging as the *Snord116del* mice were extremely small i.e. to keep the treatment/fluid from dripping out of the SNO mouse model's mouth. Also handling the animals with restraint is more stressful than our protocol of jelly-dosing. Handling is known to cause stress and this could influence the parameters of our study related to ghrelin or glucose levels. Handling was therefore kept to the minimum of care, husbandry cleaning, weighing and acclimatizing.

Though these were favourable aspects of the jelly dosing there was an altered treatment protocol within the first two weeks of the jelly treatment. At first the made up jelly was made weekly but this was altered to every second day due to the early cycling of hyperphagia of the SNO mice. As the first two weeks were only the SNO animals this jelly may have had less effect on appetite in the first litter of Snord116del mice. After this point there was a consistency in dose.

5.6.2 Further study

Due to CFE's mechanism of action in the 5-HT2C receptor, further investigations may need to address dopamine neuronal projections within the CNS, especially in regards to the mesolimbic reward circuitry which interacts with serotonin (Dichter et al., 2012). If regulatory signals are found in regards to CFE and reward systems in PWS there may be some literature to review discussing reward, satiety and OCD in humans with PWS. OCD is a major concern which could be due to interaction between dopamine and serotonin. It would be very interesting to define a study that determines if the SNO animals spinning on top of their cages has anything to do with obsessive behaviour. It may be worth taking the *Snord116* deletion mouse through behavioural experiments which include food intake, body weight, energy, levels of anxiety, self-defeat and stress in relationship to ghrelin (Lambert et al., 2011, Schellekens et al., 2015{Schellekens, 2015 #735), fat deposits and CFE administration.

As is written within the literature and supported by this work on CFE in mice, those with PWS have different 5-HT2cR messenger RNA (mRNA) isoforms (Doe et al., 2009). The full consequence of this transcriptional modification of the 5-HT2c receptor in PWS has not been fully described. It would therefore have been interesting to further this work utilizing the *HBII52 - SnoRNA115 deletion* mouse model *mbii52 - Snord115* to fill in gaps related to the regulation of the 5-HT2c receptor. Research could address the role of CFE on appetite pathways within the CNS more thoroughly determining the regulation of the 5-HT2c receptor by CFE. A viable PWS mouse model lacking the expression of mbii-52 – similar to the PWS-IC$^{+/-}$ (Doe et al., 2009, Yazdi et al., 2013), may demonstrate the consequences of CFE on altered editing of the 5-HT2cR pre-RNA using immunohistochemistry fluorescence. This further study could define if CFE stimulates the 5-HT2c receptor through regulatory hormonal processes which increase corticotrophin releasing hormone, vasopressin mRNA both within the paraventricular or if CFE interacts through the 5-HT2c receptor, through co-expression or as a ligand, perhaps binding within the neuronal pathways.

Defining a clinically efficacious capacity of decreasing appetite or compulsions in PWS is significant. Yet further as it was determined there was a stronger efficacy of CFE at a higher dose in children and adolescents. This difference in dose related to gastric motility and malabsorption is important to understand between the WT and SNO animals and the dose. It is also important to investigate the energetic aspects of this treatment now with a knowledge of hyperactivity in the animals. Studying pathways including leptin's role related to adiposity and

the CFE's role in the synthesis of ATP. This could give us more understanding related to humans with PWS. CFE"s role in thirst is important to follow through with in humans, especially if diagnosed with PWS as viscous saliva is part of the phenotype (Saeves et al., 2012). Appetite may become a response to dehydration lead by the osmolarity of electrolyte fluid mineral balance. It may be that the brain contains serotonergic neurons which project information forward toward the forebrain for the regulation of the electrolyte composition and volume.

Naturally feedback loops flow throughout the body; i.e. oxygen depletion or excessive temperature may cause sleep disorders, anxiety or seizures and sleep can determine important massages in regards to satiety (Van Cauter and Knutson, 2008). Further experiments could test if the SNO mice are more likely to eat when woken, or if their sleep is more disrupted. Taking this hypothesis further, perhaps the amount of food eaten during the SAL experiments in the SNO animals compared to WT, would have levelled out over the dark cycle past the four hours, when perhaps the WT animals would continue to eat whilst the SNO animals settled again. This may explain the discrepancy between the SAL experiment and the chronic daily results.

In PWS typical fat distribution and lean mass alterations will alter glucose sensing and appetite signalling. So too sodium depletion can create an increased appetite. Nutrient and sodium uptake in the cells etc. can also cause action over and above appetite and fluid alterations. During this process there are also gastric alterations due to metabolic function and this can even enhance OCD behaviour due to the activity of serotonin (Reis, 2007). There is an opportunity here to test this hypothesis. Importantly thirst and sodium levels may be adjusted due to serotonin or lack thereof (Harrison et al., 1997). In PWS there is much communication within the community on the difference in the consistency of mucous within the mouth and the lack of thirst in individual humans with PWS (Saeves et al., 2012). Anecdotally this has been influenced by CFE but further study must address CFE's influence on water consumption, circadian rhythms (cycles), sleep and gastric motility.

5.7 Conclusion

Investigation of appetite behaviour in the cre-mediated recombination Garvan *Snord116* deletion animal model (SNO) from an original C57BL/6 base and a C57BL/6 WT control from the same laboratory, demonstrate that CFE did not cause a significant difference in appetite in any of the SNO and WT control experimental groups. These values over the eight-week chronic treatment trial, were parallel with the animals' daily body weight measurements which also determined no significant differences in any of the treatment groups from baseline to the end of the trial period. Over the chronic trial there were no significant appetite reductions within the SNO strain due to CFE, though a significant difference was observed due to treatment CFE 100/kg/d (the highest dose), between the strains with the SNO study animals on 100CFE (n=12) demonstrating the strongest reduction in appetite against the highest increase in chow intake measured. Unexpectedly this increased appetite was in the WT-100CFE group. The faecal experiments determine that the increased appetite in the WT-100CFE was in-part due to malabsorption of diet resulting in increased HE in comparison to all other groups (P = <0.001). Also hyperactive behaviours observed in the WT mice, ingesting CFE, may have increased the need for food. In all animals' CFE had no capacity to adjust the animals' appetites during the 50% appetite deprivation

The outcomes during the appetite stimulation experiments showed many significant interactions both due to CFE and strain. For example, there were significant differences during glucose deprivation 2DG i.p. administration with strains experiencing opposite effects i.e. stimulated feeding in the WT animals and reduced feeding in the SNO strain, 100CFE (P = <0.001), 33CFE (P = <0.001) and the PLAC of (P = 0.003). Importantly the fourth appetite stimulation experiment utilizing the 5-HT2cR antagonist; SB 242084 (Kennett et al., 1997) ultimately identified the role of the 5-HT2c receptor in CFE induced reductions of appetite. As expected the experiment correctly enhanced feeding in all animals including blocking the natural serotonin levels in the PLAC groups. This determined SSRI activity due to CFE leading to a need for future study.

In regards to water appetite unusual interactions were defined between strains and between treatment especially during the four-hour MA stimulation, with lowered water appetite in all the SNO mouse treatment groups. This determines an interaction between the *Snord116* deletion and water appetite during the blocking of fatty acid oxidation.

Further different appetite behaviours, postures, adiposity and activity during the complexities of the experimental protocol demonstrated unusual activity that will enable some important characterization of this animal model related to CFE and the PWS deletion SnoRNA 116. The most interesting characteristic was during the 50% deprivation study where after two-days deprivation, near to all the SNO mice exhibited spinning behaviour on their house roofs, which was not observed in the WT animals.

There are many indications for further study. Chapter six's immunohistochemistry experiments will determine if the activity in the brains due to 2DG administration in the same animals visually represents the values recorded by the behavioural experiments, in the same animals.

CHAPTER SIX

CHAPTER SIX

IMMUNOHISTOCHEMISTRY DURING APPETITE STIMULATIONS IN THE SNORD116 MOUSE MODEL.

6. Study three

6.1 Abstract

As the first studies have demonstrated different presentations of appetite in regards to CFE in PWS, i.e. the reduced appetite experienced in the children and adolescents with PWS, to the inconsistent significant differences in appetite between the mouse model strains; this chapter is designed to investigate the modulation of appetite activity in regards to cell population signalling in the brains' CNS. To date no research has clearly defined the mechanism of CFE's interaction within hypothalamic pathways. Also important to PWS, this chapter will identify activity within appetite pathways regarding the genetic deletion of the *Snord 116* in animals.

Aim

The aim of this chapter was to determine if chronic administration of CFE at 100mg/kg/d modulates immunohistochemistry markers of appetite in the mouse brain's hypothalamus of the CNS, in the same SNO and WT mice from study two under similar basal and conditional glucose stimulation.

Hypothesis

This chapter hypothesizes that the visual representative images of immunohistochemistry cell activity of c-Fos, NPY and α-MSH expression will present similar modulation related to the results in food and thirst appetite, presented in the behavioural studies of study two (chapter five) and that these alterations due to stimulation by 2DG or the control SAL will define comparable interactions with strain and treatment CFE or PLAC.

Methods

The immunohistochemistry appetite signalling investigations were spread over four-weeks in the same animals, as in chapter five. Therefore, after ten weeks of chronic administration of CFE or PLAC the animals were randomly grouped to eight perfusions per day. For each animal on the day of experimentation - after a short period of light-cycle fasting the SNO or WT mice were voluntarily fed CFE or PLAC (dose by weight) and administered acute i.p. stimuli of 2DG, MA or the control SAL at twelve minute intervals, at ninety minutes before perfusion.

For perfusions the animals were deeply anesthetized with an intraperitoneal injection of 0.15ml sodium pentobarbitone and perfused transcardially by normal saline and paraformaldehyde (PFA) (4%) in 0.1M phosphate buffer (PB) (pH7.2). The brains were removed and cryoprotected for storage at (-20°C) until immunohistochemistry. Coronal sections of brain were cut (40μM thickness) and triple-labelled immunohistochemistry was utilized with the primary antibodies to detect early gene expression Fos protein in cell nuclei, NPY and α-MSH revealing activated neurons within the ARC, PVN and MnPO. Secondary antibody florescent stains were utilized for co-localized activity of c-Fos, the orexigenic NPY and anorexigenic αMSH neurotransmitters.

Images of the mouse brain were obtained at 40X magnification using a Nikon E400 confocal microscope and counting was defined by the investigator after consultation with two others investigators. Statistics were defined using the computer program SPSS version 23.0 for Windows. All data was determined as normally distributed with $P \leq 0.05$ as significant. Analysis of the cell activity was organized into mean ± SD per animal (n=5) , per group x eight, to give results per (strain/treatment/stimulant), per area of interest (AOI) (ARC/PVN/MnPO x 2-3 slides per AIO) and per immunohistochemistry stain (c-Fos/NPY), using channels, FITC (488) green, NPY; ALx594 (561) Red, α-MSH and Cy5 (640) Purple: c-Fos. Images were scanned from two-three brain slides per region of interest (ROI) in the mouse brain. Effect size was analysed using a two-way ANOVA.

Results

The results determined similar activity to the behavioural results from study two, though the SNO-100CFE animals had stronger activity than the SNO-PLAC, they were both much lower than the WT animals on CFE and PLAC. In the SNO- PLAC animals though NPY activity in

both 2DG & SAL groups had decreased appetite compared to SNO-100CFE, the α-MSH activity in the SNO-100CFE created a stronger inhibitory signal. Even so overall brain slice images, none of the α-MSH co-localization signals were significant. C-Fos and NPY signalling differences were strongly significant in the ARC, between strains, SNO-PLAC-2DG: 49.6 ± 16.68; WT-PLAC-2DG: 218.4 ± 55.38, (P = < 0.001) and the PVN: SNO-PLAC-2DG: 51.0 ± 15.84; WT-PLAC-2DG (n=5) 138.2 ± 49.17, (P = 0.005). In the MnPO the lowest parameters for thirst were in the SNO-PLAC-2DG with a reduced fluid consumption seen in both c-Fos and NPY. In the WT animals those on 100CFE had the lowest thirst. Reductions in cell activity of appetite due to CFE was only minimally noted as in the behavioural studies.

Conclusion

The results of the immunohistochemistry experiment present confirmation of much of the behavioural data with significant differences mainly between the strains SNO and WT. Ingestion of CFE at 100mg/kg/d compared to PLAC, during stimulation by 2DG or the control SAL, determined only minimal reductions in appetite signalling, with many opposite levels of c-Fos and NPY activity between strains. The lowest food appetite was in the SNO-PLAC animals, ninety-minutes after glucose deprivation by 2DG injections. At the other end of the spectrum the highest levels were identified in WT-PLAC group at ninety-minutes after 2DG. Therefore, as in the earlier behavioural experiments, glucose deprivation lowered the indicators of appetite in the SNO animals. This was also the case in the SNO animals ingesting CFE though the activity in the brain was different with a stronger indication of α-MSH co-localization. Though overall groups, there were no significant α-MSH levels in the ARC and PVN of the CNS. The MnPO immunohistochemistry levels for thirst followed the markers for food appetite.

6.2 Introduction

6.2.1 Immunohistochemistry study in animals

Nucleus integration of chemical signals alter the functional default in a cell through many inhibitory and excitatory signals, eventually leading to a single message; on or off. These continual CNS messages inhibit or excite electrical activity which adjust the coordination of homeostatic activity (Kandel et al., 2000). These experiments identify the activity of hunger

and thirst created by inhibitory and excitatory messaging as natural responses but also question the activity in the SNO animals in regards to cell population signalling.

The behavioural studies of chapter five (study two) demonstrated differences in appetite and thirst in the SNO and WT mouse models due to CFE and the appetite stimulants which warranted further mechanistic investigations. This chapter's study – study three - compares the neural activity of anorexigenic and orexigenic peptides to address these differences. The protocol chosen was due to the literature on the CNS glucose/insulin sensing (Quarta and Smolders, 2014), oxidation of glucose (Berthoud and Levin, 2012), function mapping of cells in the brains in mice (Cone, 2005, Cowley et al., 2001, Chen et al., 2004). This is a standard procedure for visual neuronal confirmation of behavioural appetite markers and importantly this study also examines outcomes relative to the PWS SnoRNA deletion which is suspected to create a dysfunction in appetite related hypothalamic signalling. Unfortunately, for the immunohistochemistry study there was only enough animals, money and time, to confirm some of the more significant behavioural interactions. Therefore, not all behavioural results from the complex appetite signalling experiments of chapter five were confirmed. Only the most significant results were chosen which mainly includes the mechanistic activity of CFE, due to the stimulant 2DG. This choice for the immunohistochemistry protocol was to answer three specific questions important to outcome of this thesis:

1. Does the treatment CFE alter cellular neural appetite and thirst activity in known pathways within the CNS?
2. Does the *Snord116 deletion* strain of mouse alter the levels of neural activity in these CNS pathways?
3. Does glucose deprivation by an i.p. injection of the appetite signalling reagent 2DG, alter the outcome for each of the above, confirming the behavioural activity of earlier work.

Related to the design, it was hypothesized that the dose response to CFE 100mg/kg/d would give a more active neuronal result, than the lower dose 33mg/k/d. The design was concerned with the opposite reactions in strain during the four-hour glucose stimulation period after i.p. injection of 2DG (Jordan et al., 2010, Farooqi and O'Rahilly, 2009, Dubern and Clement, 2012) and observing the appetite activity and the specific areas of interest (AOI), the ARC and PVN of the hypothalamus. Within the design, the neurotransmitters for isolation and comparison were NPY and α-MSH. These are important in ascertaining orexigenic and anorexigenic

activity (Fraley and Ritter, 2003, Cowley, 2003, Nuzzaci et al., 2015, Stevanovic et al., 2014) and c-Fos activity (Ueta et al., 1995, Pereira-Derderian et al., 2016). Further the MnPO was chosen as a representative of thirst in the lamina terminalis (Zardettosmith et al., 1993, McKinley et al., 2004) also associated with increased activity of NPY (Pich et al., 1992).

Though all the animals were perfused (n=72), the experimental protocol utilized immunohistochemistry detection in response to CFE chronic administration in only sixty of the harvested brains - after the death of the animals. Fourty-eight animals received 2DG stimulation or the control SAL and the other twenty-four mice were acutely administered the appetite signalling reagent MA or the control SAL. This fatty acid signalling, appetite stimulant was specifically added to the protocol to indicate if further study with MA was warranted. Many of these latter animals were on the dose of 33mg/kg/d before perfusion but their brains were harvested and placed in frozen storage for further study.

Therefore this study (study three) determined and compared the mechanism of neuronal activity in the brains of the animals, using c-Fos, NPY and α-MSH with secondary fluorescent staining in both the *Snord116* deletion and WT mouse models chronically ingesting CFE, or the control PLAC, during appetite signalling after glucose deprivation, utilizing 2DG (Li and Ritter, 2004) against the control vehicle saline.

The randomized groups were given an even allocation of, parentage, gender and treatment relative to the appetite signalling/saline per the perfusion protocol. The protocol allowed for a time of recovery after the deprivation studies and a short period of fasting in the morning before the acute stimuli. After treatment and stimuli, the different groups of animals were deeply anesthetized with an intraperitoneal injection of 0.15ml sodium pentobarbitone and perfused transcardially by normal saline and paraformaldehyde (4%) in 0.1M phosphate buffer (pH7.2). The brains were harvested and snap frozen and transferred to the Melbourne Brain Centre for immunohistochemistry, where they were cryoprotected for storage at (-20°C), ready for sectioning by cryostat until immunohistochemistry.

The brains were removed and coronal sections of brain were cut (40μM thickness) and triple labelled immunohistochemistry was utilized with the primary antibody to detect as early gene expression Fos protein in cell nuclei, revealing activated neurons within the ARC, PVN and MnPO (Ueta et al., 1995). The brain slice chemical-coding was labelled by administering

chemical co-localization of the protein product of the immediate early gene c-Fos, (Rabbit antic-Fos) primary in the nucleus of activated neurons. The primary fluorescent antibody labelled cellular c-Fos activity (to sheep), NPY (Mouse) and α-MSH (Rabbit), which was then co-locally labelled by secondary labelling c-Fos Donkey anti sheep 647 (Purple) (Alexa Fluor® 647) (Overstreet-Wadiche et al., 2006), adding orexigenic & anorexigenic peptides, through the secondary antibody detecting the presence of NPY: diluent at 1:40, Donkey anti Mouse 488 (Green) or α – MSH, Donkey anti-Rabbit 561 (red). These extremely bright fluorescent labels were chosen to limit cross-reactivity to each of the other bovine mouse, rabbit and sheep serum proteins.

The immunohistochemistry images were scanned and viewed on a Nikon A1 confocal program 2013. The microscopy was concerned with determining cell activity within the CNS. The Region of Interest (ROI), ROI-2 - Bregma 1.10 mm to -2.92 mm, striatum, hypothalamus, midbrain and ROI-4 - Bregma -3.80 to end of the cerebellum regarding thirst in the lamina terminalus (OVLT) were chosen for viewing. The most appetite activity found for counting was in ROI two, the ARC and PVN of the hypothalamus and MnPO of the lamina terminalus, in both animal strains, with and without treatment. The immunohistochemistry secondary fluorescent antibodies had been prepared from affinity-purified antibodies to provide extraordinarily bright conjugates. Images have been presented in chapter six's results section with details as to the counting method.

After visually identifying the best ROI for viewing and the AOI for scanning, the results were statistically defined by the investigator counting the cell activity in the ARC and PVN of the hypothalamus and MnPO of the lamina terminalus and defining the mean ± SD per animal from two-three brain slides per animal, with two-three images taken per AOI. These results were grouped as data for statistical analysis on the computer program SPSS version 23.0 for Windows. Effect size was analysed using a two-way ANOVA and the p value of equal to or less than 0.05 ($p \leq 0.05$) was considered significant.

6.3 Methods and materials

6.3.1 Preparation

6.3.1.1 Preparation for immunohistochemistry experimental protocol

Immunohistochemistry ethics was once again gained through the HFI AEC under the submission numbered 14-081-FINMH. The IBC approval for GMO's was approved by a separate application through the FINMH and confirmation of Animal ethics. Transportation of the tissue was gained through VU AEC. The immunohistochemistry appetite signalling investigations were spread over a four-week period. The animals were consistently housed in SC, which involved a temperature-controlled (21°C) mouse facility with a 12-h light:dark cycle (lights on 0600–1800 h). After the protocol of chapter five - at sixteen weeks of age – twenty-two weeks after birth, the animals had been given over a week to recover from the 50% deprivation experiments. This was the last experimental protocol with the animals so measurement of food and fluid ingested was not necessary. Leading up to the perfusions all single housed animals received over and above their daily allowance of food prior to the investigations, so as they were not stressed during the experiment.

6.3.1.2 Preparation in the Animals

On the day of the experimental protocol at 16 weeks, food (not water) was removed four hours before administration and each animal was weighed to receive their relevant dose and treatment. In this experimental procedure the appetite signalling injections were targeted to be received ninety minutes before the perfusion of the mice followed by harvesting of the mouses brains. The protocol for experimentation targeted the appetite signalling stimuli as in the earlier experiments utilizing 2DG and saline. MA investigations were in a smaller number of animals relative to further study. The groupings were focused around n=60 animals for 2DG, MA and saline injections

The animals were weighed on a balance (Sartorius AG Göttingen Germany TE412) to determine the dose of the treatment, stimulant and anaesthetic for the day. The specified treatment CFE at 100mg/kg/d or PLAC was presented within the cages as per their groupings, for voluntary administration. Ingestion was confirmed by the investigator.

At ninety minutes before the perfusion the allocated animals received an i.p. injection of reagents: a) 2DG, glucoprivic signalling (induced by i.p. injection of 2-deoxyglucose 400mg/kg or 10mg/25g mouse), (b) MA, fatty acid signalling (induced by i.p. injection of beta-mercaptoacetate 100mg/kg or 2.5/25g mouse) or c) SAL, as a control vehicle of sterile saline at an allocated volume. These i.p. injections were at exactly twelve minutes apart, decided by a randomized schedule for the injections. The immunohistochemistry groupings were as per table 16.

Table 16 Stimulant groups for the Immunohistochemistry Study Three

CFE administration, i.p injection food signalling groupings for perfusions.			
Week 16		Neural expression at 90 mins acute α - MSH & NPY	
Signalling reagents	No.	Treatment group (same animals as in study2)	No.
a) Glucoprivic sig. 2DG	24	SNO100CFEmg/kg WT 100CFEmg/kg SNO PLAC WT PLAC	6 6 6 6
b) Fatty acid sig. MA	12	SNO100CFEmg/kg WT 100CFEmg/kg SNO PLAC WT PLAC	3 3 3 3
c) Saline	24	SNO100CFEmg/kg WT 100CFEmg/kg SNO PLAC WT PLAC	6 6 6 6
Total	60		60

Table 16. Study three groupings for the perfusion of the animals relative to markers of expression in neuronal pathways. 2DG - 2-Deoxy-D-Glucose; MA - sodium mercaptoacetate, No. – number; α - MSH – alpha - melanocortin stimulating hormone & NPY – neuropeptide Y.

Similarly, the animals of mixed gender receiving the chronic treatment of CFE 33mg/kg/d over the past ten weeks, were administered an i.p. injection of either MA 100mg/k (2.5/25g mouse) in the SAL vehicle <1ml (n=12) or SAL vehicle, <1ml (n=12) for further study. Food restriction and the antagonist SB 242084 were not involved.

6.3.1.3 Preparation for Intraperitoneal injections and perfusions

The intraperitoneal injections were timed at twelve-minute intervals, given ninety-minutes before the perfusion. All the protocols timelines were recorded for future interpretation. These included when the food had been retracted, the jelly treatment allocation, the animal injection of 2DG, MA or saline and the amount and the allocated time for the pentobarbitone injection for the death of the animal. All the animals were held away from the perfusion room to reduce stress until their particular time of death. The lab and tools were organized for the perfusions into three areas: injection, perfusion (fume hood) and brain harvesting area. After ninety minutes had past, from the first schedules injection - giving the stimulant ninety minutes to activate the appetite network in the brain's CNS - the animals were taken to the perfusion room at 12 minute intervals.

6.3.2 Perfusions

6.3.2.1 Methods for perfusion

The solutions for the perfusions were made as per (Appendix Kb). One at a time at twelve minute intervals in order of their treatment, the mice were deeply anesthetized between 90100mins minutes with an i.p. injection of sodium pentobarbitone (*Nembutal* ® sodium 5-ethyl5-(1- methyl butyl) barbiturate), (dose - 80mg/kg) and perfused transcardially by normal saline and PFA (4%) in 0.1M phosphate buffer (pH7.2).

Our experimental procedure followed the SOP for a vascular perfusion in an anesthetized animal. The sixteen-week old mice were weighed to the nearest 0.1 gram and deeply anesthetized at between ninety to one hundred minutes after the signalling stimuli with an i.p. injection of sodium pentobarbitone (dose - 80mg/kg). Example: A mouse weighing 20.0 grams receives 0.8mg of the anaesthetic mixture injected intraperitoneally. After the animal dropped in their box, they were assessed for reflexes by a toe pinch. When the mouse was unresponsive and the reflex was absent they were transferred from the injection area to the chemical fume hood for perfusion. They were secured in an open supine position (lying on the back with their face upward) on a Styrofoam work surface inside the fume hood with the forepaws and hind paws splayed. The transcardial perfusion was performed by making an incision at midline through the skin in the thoracic with surgical scissors. Then from just under the xiphoid process two additional skin incisions were laterally made

along the base of the ventral ribcage. This created two flaps of skin, which were pinned back rostrally and laterally to expose the thoracic field. Once this was in view the investigator grasped the cartilage of the xiphoid process - without cutting the organ - by using blunted forceps. This was then lifted to insert sharp scissors which were used to cut through the thoracic musculature and ribcage. An incision was made between the breastbone and medial rib insertion points to separate the diaphragm from the chest wall. The incision extended rostrally, reaching the level of the clavicles an18G X 25.4mm needle was fixed in place to hold the ribcage laterally. This procedure exposed the heart for perfusion. A ventricular catheter was perfused through the left ventricle by making a 1-2mm incision in the left ventricle

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6.3.2.2 Harvesting the brains.

The gently freed brain was then placed in a vial with ten times the amount of fixative (PFA for 1-2hrs). After which time it was washed three times in PB solution with a gentle swirling motion and placed in sucrose (20gms & 100mils of PB), at ten times the volume of the brain. The closed vials were stored for up to two weeks in a -4° fridge.

6.3.3 Immunohistochemistry

6.3.3.1 Preparation for immunohistochemistry

Preparation for immunohistochemistry involved moving the transcardially perfused mouse brains from their positions floating in sucrose, to snap freezing them for slicing by a cryostat as per the following section. The mouse brain stereotaxic coordinates for slicing coronal sections were defined for both the *Snord116del* mouse brains and WT- C57BL/J6 control mouse brains, by examining the atlas for the C57BL/J6 strain adult 'Mouse Brain Atlas'. Bregma and Lamdoid points were identified for ROI immunohistochemistry staining. The structures chosen were identified by the literature as important in the appetite behaviour in both animals and humans. Blocking the structures both Bregma/Lambda points of intersections in the C57BL/J6 strain adult mouse brain were classified as the "best-fit" centreline curve along the coronal and

Lamdoid structures. These coordinates denote the plate's anteroposteria distance from Bregma and the dorsoventral distance (defined in milometers) from the plane which passes horizontally through

the single cross point of Bregma and Lamda on the skulls surface. Coordinates were defined by ROI 1: Bregma 4.28 mm to 1.10 mm (OB to frontal cortex), blocked off and stored at -20C for future analysis. ROI 2 Bregma 1.10 mm to -2.92 mm (Striatum, hypothalamus, midbrain) 1:4 series = approx 25, 40□m sections per series. ROI 3 Bregma -2.92 to -3.80 mm (midbrain SN) 1:2 series = approx 11, 40□m sections per series and 1:3 series = approx 7, 40□m sections per series (to be mounted for future analysis). ROI 4 Bregma -3.80 to end of the cerebellum (OVLT) 1:4 series = approx. 26, 40□m sections per series sections and 1:8 series = approx 13, 40□m sections per series. The eventual region used was ROI 2. The distance in millimetres was slightly reduced for each from the midline, in the case of the smaller SNO animal.

Note: other areas were kept for further research.

6.3.3.2 Immunohistochemistry labelling

Triple-labelled immunofluorescence was carried out by first immersing the brains in 30% sucrose by weight (w/v) in 0.1M PB for 72 h. Next the brains were rapidly frozen by submersion in isopentane cooled to approximately -80 °C on dry ice. To slice the brains for staining, free-floating coronal sections of 40 µm thickness were cut on a Leica Microsystems CM1850 cryostat relative to the specified ROI above. The sections were placed into cryoprotectant solution (30% ethylene glycol and 15% sucrose in PB) and stored at -20 °C prior to staining.

Primary antibodies were diluted in antibody diluent (1% NDS and 1% Triton X-100 in PB) as follows: Rabbit anti-c-Fos primary 1:2000, anti-NPY 1:1000, (Abcam, Anti-Neuropeptide Y antibody: ab112473 Neuropeptide Y, 100 µg x 4, Mouse monoclonal), and anti-MSH 1:10000 (Merc-Millapore, Anti-Melanocyte Stimulating Hormone α Antibody, 50ul x 4). Sections were incubated with primary antibodies over two nights at 4 °C with agitation. Sections were then washed in PB (3 x 10 minutes). The secondary stains were administered to the slides on three separate days, which meant that these time-points (1, 2 & 3), needed slightly different confocal settings to view the same tone and brightness for each slide. Even so all time-points followed the same procedures: before staining the sliced sections were washed in PB (2 x 10 minutes) and were blocked for 30min at room temperature with agitation in 10% normal donkey serum (NDS) and 1% Triton X-100 (Sigma Aldrich) in PB. Secondary antibodies were diluted in antibody diluent at 1:400 NPY: - Donkey anti Mouse/rabbit 488 (Green) (Alexa Fluor® 488 AffiniPure Donkey Anti-

Mouse) IgG (H+L), (Luo et al., 2015) and alpha-MSH: Donkey antiRabbit IgG (H+L), secondary antibody, (red) (Alexa Fluor® 594 conjugate a21207)

(Purkartova et al., 2014) and c-Fos Donkey anti sheep 647 (Purple) (Alexa Fluor® 647) (Overstreet-Wadiche et al., 2006). Sections were incubated with secondary antibodies (3hrs) at room temperature. Sections were washed in PB (3 x 10 minutes) and were mounted as per ROI 2 order for slices on Superfrost Plus slides (Hurst Scientific) in mounting medium ProLong Diamond Antifade mountant (Life Technologies) with #1.5 coverslips (Hurst Scientific) applied. The edges of coverslips were sealed with a clear nail varnish. The slides were then transferred in a cold container to Western Centre for Health Research and Education (WCHRE) Sunshine Hospital Victoria University, St Albans laboratory on the fourth floor of WCHRE.

6.3.3.3 Imaging the brains

Images of the mouse brain were obtained using a Nikon A1desktop confocal 2013 NIS Elements AR4.1300 64-bit Nikon-provided software and transferred to an Olympus FV1000 inverted confocal microscope and Olympus Fluo View Software for counting. The measurement depth of the images optical resolution was between 4600 – 4900. The settings for background and brightness were set at ranges as similar as possible for the three different staining batches (1,2 &3). Overall >1,400 images dilutions of antibodies resulted in different intensity in staining which was visualized only by using specific brightness/background setting ideal for each antibody, defined in table.17 below. Where possible, the investigator made sure the tones were similar.

Table 17 Nikon A1 Desktop Confocal Settings

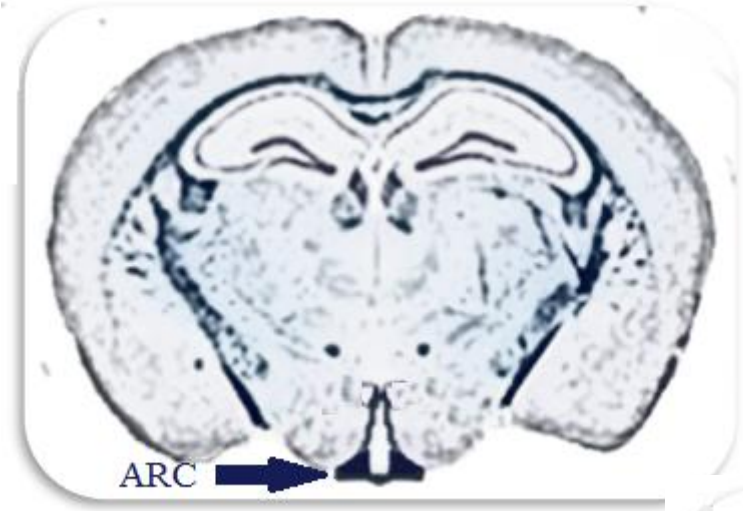
Table Secondary antibody confocal settings

Fluorescent antibody	Background settings	Brightness settings
Primary antibody, c-Fos 647 (Purple)	90 – 130	1.50 - 2.00
Secondary Antibody, NPY 488 (Green)	15 - 40	0.30 - 0.70
Secondary Antibody, α-MSH 594 (red)	70 - 100	1.00 - 140

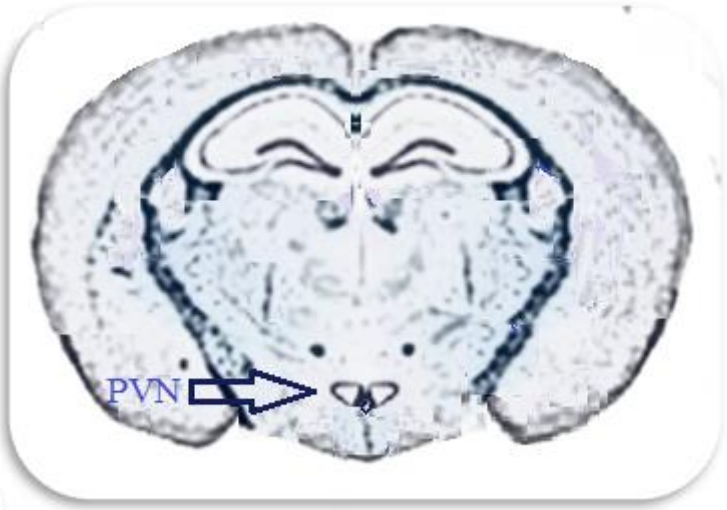
Table 17, ranges for the confocal settings due to the stains being carried out over three time points for the secondary antibody staining.

This experiment revealed the chemical-coding of neuronal cells activated by the stimuli in *Snord116del* mouse model and WT controls: (n=60 x approx. 36 brain slices per animal of ROI). The scan areas of the AOI were 512□m square. The 40X magnification was scanned at a speed of 0.041 frames per second, making 24.1 seconds per scan. Layered Z stacks were also captured 5.000□m. 0.250□m x 11steps, at a range of 40□m. The count was acquired from the Z stack centre line and all other images - five layers above and five layers below - were set as the values i.e. ended where the image disappeared 40□m thickness. Scan areas are as per figures 21 a, b and c, from the approx. twelve brain slices per ROI. 1, where the images were scanned for activity after testing ROI 1 & 2.

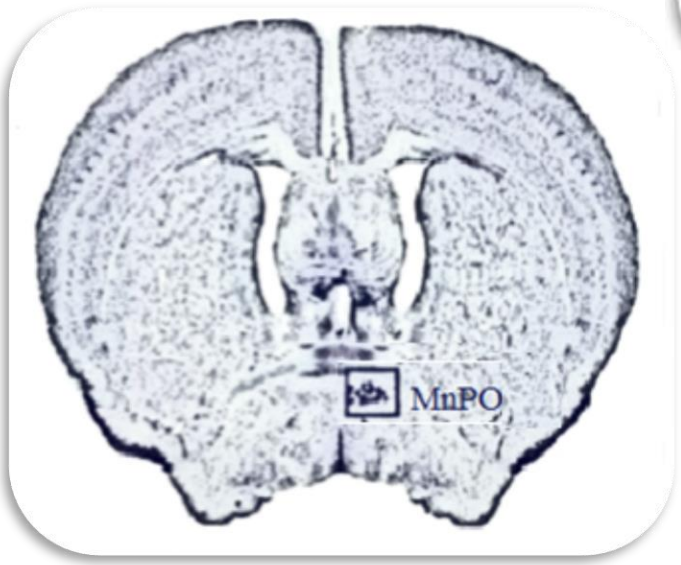
Note: in the images where the cell had unusual repeated staining by NPY and α-MSH (on the same cell), the next stacks above and below were observed from the Z stack to check the label wasn't captured from a cell underneath or above the centre counted image.



a) ROI 2, image of mouse brain AOI 1, ARC of the hypothalamus.



b) AOI 2, where the arteria part of the hypothalamus holds the PVN



c) AOI 3, image where the LSI is present. The square shows the scanned area which was the scanned part of the medial area of the MnPO: medium preoptic.

Figure 21 Areas of Interest from the Hypothalamus

The images define the ROI: Region of Interest 2 from Bregma 1.10 mm to -2.92 mm (Striatum, hypothalamus, midbrain) in coronal sections of the mouse brain and the AOI: area of interest, a) ARC: arcuate nucleus of the hypothalamus b) PVN: paraventricular nucleus of the hypothalamus c) MnPO: median preoptic nucleus of the lamina terminalis, clearly recognizable by being the strongest point of activity under the LSI: lateral septal nucleus intermediate part.

Figure 21 Areas of Interest from the Hypothalamus

The images define the ROI: Region of Interest 2 from Bregma 1.10 mm to -2.92 mm (Striatum, hypothalamus, midbrain) in coronal sections of the mouse brain and the AOI: area of interest, a) ARC: arcuate nucleus of the hypothalamus b) PVN: paraventricular nucleus of the hypothalamus c) MnPO: median preoptic nucleus of the lamina terminalis, clearly recognizable by being the strongest point of activity under the LSI: lateral septal nucleus intermediate part.

6.3.4 Statistical analysis

Statistical analyses were performed using the IBM SPSS version 23.0 for Windows. The images were grouped as per table 16 (n=5 mice x 8 groups: ARC x 2-3 per mouse, PVN x 2-3 per mouse). Copies per image (x 7; c-Fos / NPY / α-MSH / NPY & c-Fos / α-MSH & c-Fos / NPY & α-MSH / all markers). Importantly, though the groups for perfusion involved six animals with mixed gender, the immunohistochemistry brain slices for counting only numbered five per group. This was due to defining the groups for equal variance as in some instances, the first animal of the group was utilized as a test animal for c-Fos and florescent experimentation. Also a couple of the animals were not perfused as well as was expected, therefore they were deleted from the groups counting. To evenly investigate all the other group's data, the sixth animal's data sets - of the other three groups - were deleted.

The analysis of the chemical-coding of neuronal cells activated by the stimuli 2DG and saline were determined by counting and quantification of the fluorescent neuronal cells within the mounted mouse brains slices as per images figures 23-29. The mean and SD per animal, was transferred to SPSS for statistical analysis. The treatment and appetite signally analysis utilized a two-way ANOVA. Normality with no missing values was met in the groups (n=5), with two dependent variables of strain (SNO and WT), two dependent variables of treatment (100CFE/kg/d & PLAC) and two dependent variables regarding the appetite stimulation (control - SAL and the appetite stimulant - 2DG). There were three independent variables of activity (c-Fos, NPY and α-MSH). The results were defined with strain, treatment and stimulation as factors of variance. This was followed by Post Hoc, Turkey's tests to specifically pinpoint significance related to the eight group variations (Table 16). The value of $P \leq 0.05$ was considered as significant. All mean and SD are tabled in Appendix K. c/ and examples of activity are presented in the figures 33 - 40

6.4 Results: Immunohistochemistry

The results of the immunohistochemistry investigations related to inhibitory and excitatory pathways of the hypothalamus have determined the levels of c-Fos activity and florescent of circulating neurotransmitters NPY and α-MSH activity in brain slices from three AOI in the hypothalamus of the CNS. Two – three scans per animal from the ARC and PVN, establishing neuronal food appetite activity and one image from MnPO per animal as a representative marker of water appetite.

6.4.1 Appetite activity

The SNO mice presented with significantly lower appetite than the WT mice. On first glance the SNO control PLAC animals did not have the higher appetite compared to the SNO-100CFE. Yet this was balanced out when analysing all the food appetite data presenting the complexity of the appetite pathway, Tables 18-20 i.e. ARC, c-Fos SAL: SNO-100CFE 118.8 ± 28.41; SNOPLAC 51 ± 15.84; NPY SNO-100CFE 77.2 ± 43.82; SNO–PLAC 24 ± 12.94; α-MSH SNO100CFE 16.2 ± 15.99; SNO-PLAC 3.8 ± 3.11 (NS); PVN, c-Fos SAL: SNO-100CFE 79.6 ± 29.19; SNO-PLAC 97.2 ± 43.05; NPY SNO-100CFE 53.8 ± 28.08; SNO–PLAC 42.8 ± 27.66; α-MSH SNO-100CFE 9.2 ± 7.05; SNO-PLAC 1.75 ± 2.19 (NS); The α-MSH activity created a stronger inhibitory signal and the pathway through to the PVN adjusted the original strength of the c-Fos activity. Therefore, this is a similar in reaction to the behavioural markers. The not significant results are mainly due to the very high SD.

Overall, none of the α-MSH co-localization signals were significant in the ARC and PVN (Table 18 & 19). The SNO-PLAC-SAL controls had the lowest α-MSH co-localization to cFos. The highest α-MSH co-localization with c-Fos was in the ARC of the WT-100CFE-SAL & SNO-100CFE-2DG. Significant c-Fos and NPY signalling differences were mainly between strains (Table 18, 19 & 20). In the ARC (Table 18), the c-Fos activity was significantly different between strains 2DG, SNO-PLAC 49.6 ± 16.68; WT-PLAC 218.4 ± 55.38, (P = < 0.001). Though the

standard deviation was high in both c-Fos and NPY activity there was clearly a stronger signal in the WT animals 2DG NPY: SNO-PLAC 21.0 ± 15.93; WT-PLAC 159.8 ± 65.53 (P = < 0.001).

The NPY ARC activity in WT-PLAC group was significantly higher than all other groups (Table 18 and graph Figure 22). These results confirmed that the WT-PLAC group, exhibits the highest appetite when stimulated by 2DG which also indicates inhibitory activity of CFE.

In the PVN c-Fos activity (Table19), the results between the strain groups (n=5) were also significant 2DG; SNO-PLAC 51.0 ± 15.84; WT-PLAC 138.2 ± 49.17, (P = 0.005). The 2DG NPY signal was also significant SNO-PLAC 24.0 ± 12.94; WT-PLAC 76 ± 18.07 (P = < 0.001). This confirmed the behavioural markers of appetite between strains. It also confirmed the difference in strain appetite, where in the SNO animals 2DG was less stimulating of appetite than SAL and in the WT animals the expected stimulation of appetite by 2DG increased appetite, more than SAL.

In regards to the WT animals on 100CFE the c-Fos activity (n=5 x 2-3 scans) in the ARC due to 2DG were: WT-100CFE 146.2 ± 43.56; WT-PLAC 218.4 ± 55.38, (P = 0.04). However, the increased activity of NPY in the WT animals on PLAC did not result in significance, nor the cFos or NPY in the PVN (Table 19). These groups c-Fos activity did show significance to the SNO animals tables 18. The graphs in figure 22 visually present the values in the table.

Table 18 Group Comparisons during Stimulation in Arcuate Nucleus

Table. Pairwise comparisons of average counts of cells for florescent c-Fos, NPY and α-MSH activity in the arcuate nucleus of the Snord116 deletion and WT control mouse brain sections.

ARC	c-Fos		Pairwise comparison	
c-Fos	**Mean**	**SD**		***P value***
SNO-100CFE -2DG	88.2	± 19.18	All SNO groups	(NS)
SNO-100CFE-SAL	118.8	± 28.41	SNO- PLAC- SAL	0.08 (NS)
SNO-PLAC-2DG	49.6	± 16.68	WT-PLAC-2DG	<0.001**
SNO-PLAC-SAL	51	± 15.84	WT-PLAC-SAL	0.02*
WT-100CFE-2DG	146.2	± 43.56	WT-PLAC-2DG	0.04*
WT-100CFE-SAL	138.6	± 24.13	SNO-PLAC-SAL	0.006**
WT-PLAC-2DG	218.4	± 55.38	Sig. to all other groups	0.04* - <0.001**
WT-PLAC-SAL	128.6	± 40.12	WT-PLAC-2DG	0.005**
NPY				
SNO-100CFE -2DG	51	± 37.51	All SNO groups WT-PLAC-2DG	(NS) 0.005**
SNO-100CFE-SAL	77.2	± 43.82	All other groups	(NS)
SNO-PLAC-2DG	21	± 15.93	WT-PLAC-2DG	<0.001**
SNO-PLAC-SAL	24	± 12.94	All SNO groups WT-PLAC-2DG	(NS) <0.001**
WT-100CFE-2DG	109.2	± 42.84	SNO-PLAC-2DG	0.04*
WT-100CFE-SAL	62.6	± 33.9	WT-PLAC-2DG	0.02*
WT-PLAC-2DG	159.8	± 65.53	Sig. to above, NS. to SNO-100CFE-SALWT-100CFE-2DG & WT-PLAC-SAL	0.01* - <0.001**
WT-PLAC-SAL	98.6	± 47.41		(NS)
α-MSH				
SNO-100CFE -2DG	28.2	± 10.47	All groups	(NS)
SNO-100CFE-SAL	16.2	± 15.99	All groups	(NS)
SNO-PLAC-2DG	16.8	± 12.11	All groups	(NS)
SNO-PLAC-SAL	3.8	± 3.11	All groups	(NS)
WT-100CFE-2DG	20	± 14.07	All groups	(NS)
WT-100CFE-SAL	30.8	± 24.24	All groups	(NS)
WT-PLAC-2DG	20.8	± 11.88	All groups	(NS)
WT-PLAC-SAL	17.6	± 9.15	All groups	(NS)

Table 18 ANOVA Post Hoc pairwise comparisons with estimated marginal means and SD - standard deviation. Results of immunohistochemistry cell counts for c-Fos:- Fos-like early gene expression, NPY: neuropeptide–Y and α-MSH: alpha - Melanocyte-stimulating hormone, in brain slices from the ARC: - arcuate nucleus of the hypothalamus of two mouse strains: SNO: - Garvan *Snord116del* (n=5) and WT: - C57BL/6 wild type, (n=5) per group. Animals were ingesting either chronic treatment 100CFE: - *Caralluma fimbriata* extract, at 100mg/kg/d or PLAC: – placebo of maltodextrin/cabbage leaf with appetite signalling reagents, 2DG: - 2-deoxy-glucose compared to the control of SAL: - saline

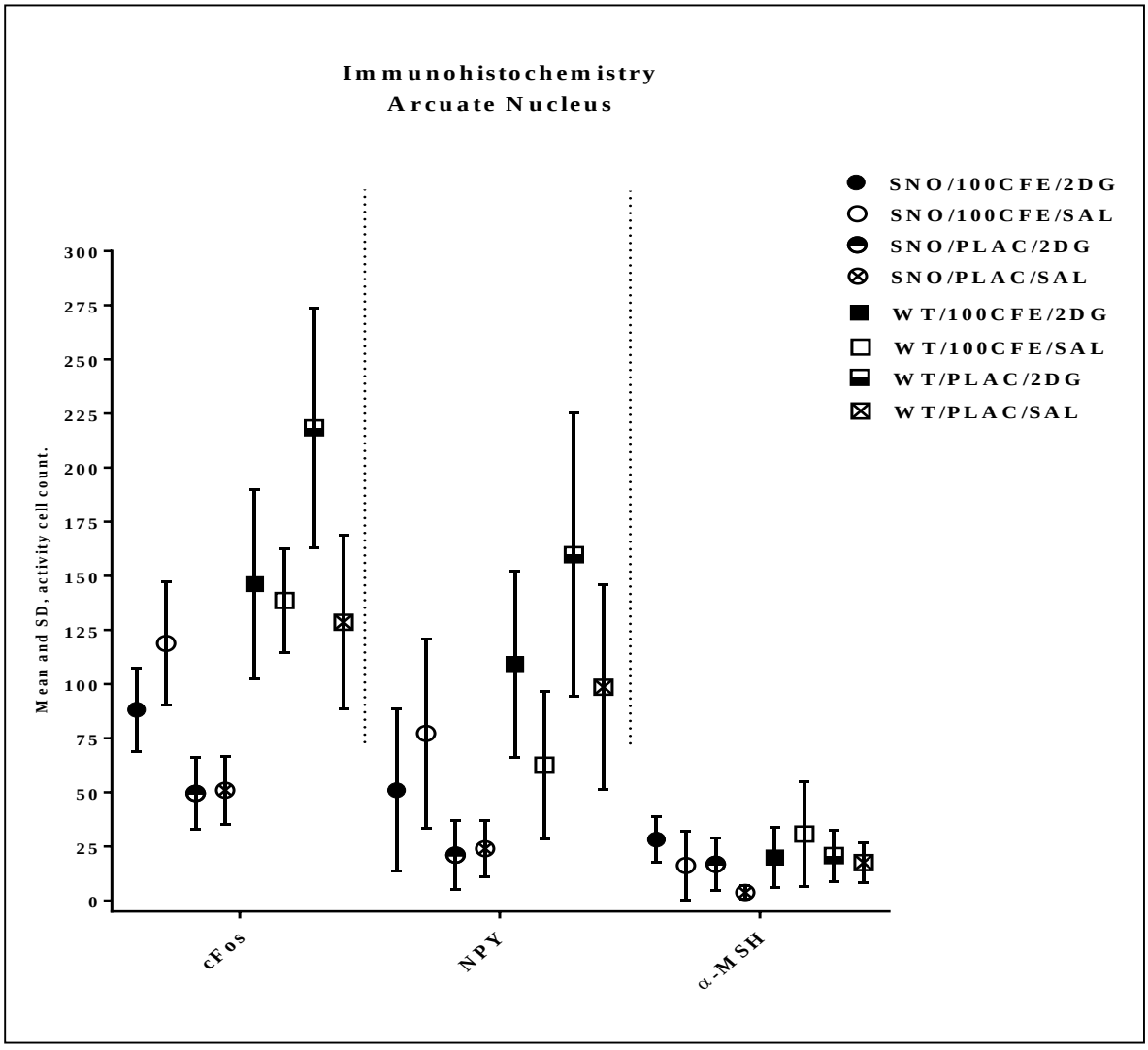


Figure 22 Comparisons of Activity in the Arcuate Nucleus

Graph of estimated marginal means and SD - standard deviation for immunohistochemistry cell counts of c-Fos:- Fos-like early gene expression, NPY: neuropeptide–Y and α-MSH: alpha – Melanocyte stimulating hormone, in brain slices from the ARC: - arcuate nucleus of the hypothalamus of two mouse strains: SNO: - Garvan Snord116del (n=5) and WT: - C57BL/6 wild type, (n=5) per group. Animals were ingesting either chronic treatment 100CFE: - Caralluma fimbriata extract, at 100mg/kg/d or PLAC: – placebo of maltodextrin/cabbage leaf with appetite signalling reagents, 2DG: - 2-deoxy-glucose compared to the control of SAL: - saline

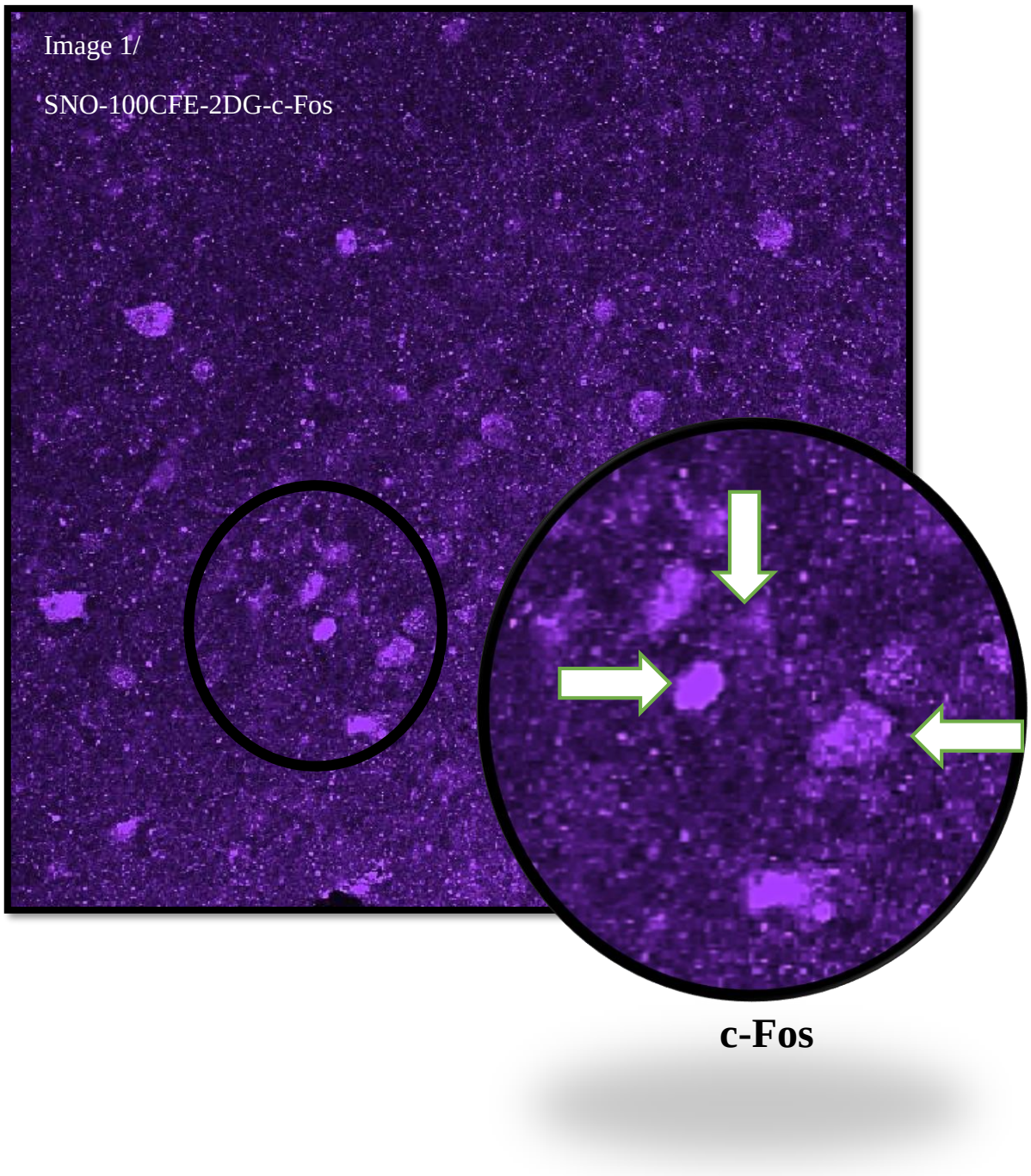


Figure 23 Immunohistochemistry c-Fos Labelling

C-Fos (purple):- Fos-like early gene expression, mean 88.2± 19.18, in brain slices from the ARC: - arcuate nucleus of the hypothalamus in SNO (n=5), image representative of *Snord116del* mice, ingesting chronic treatment 100CFE: - *Caralluma fimbriata* extract, at 100mg/kg/d, with appetite signalling reagents, 2DG: - 2-deoxy-glucose.

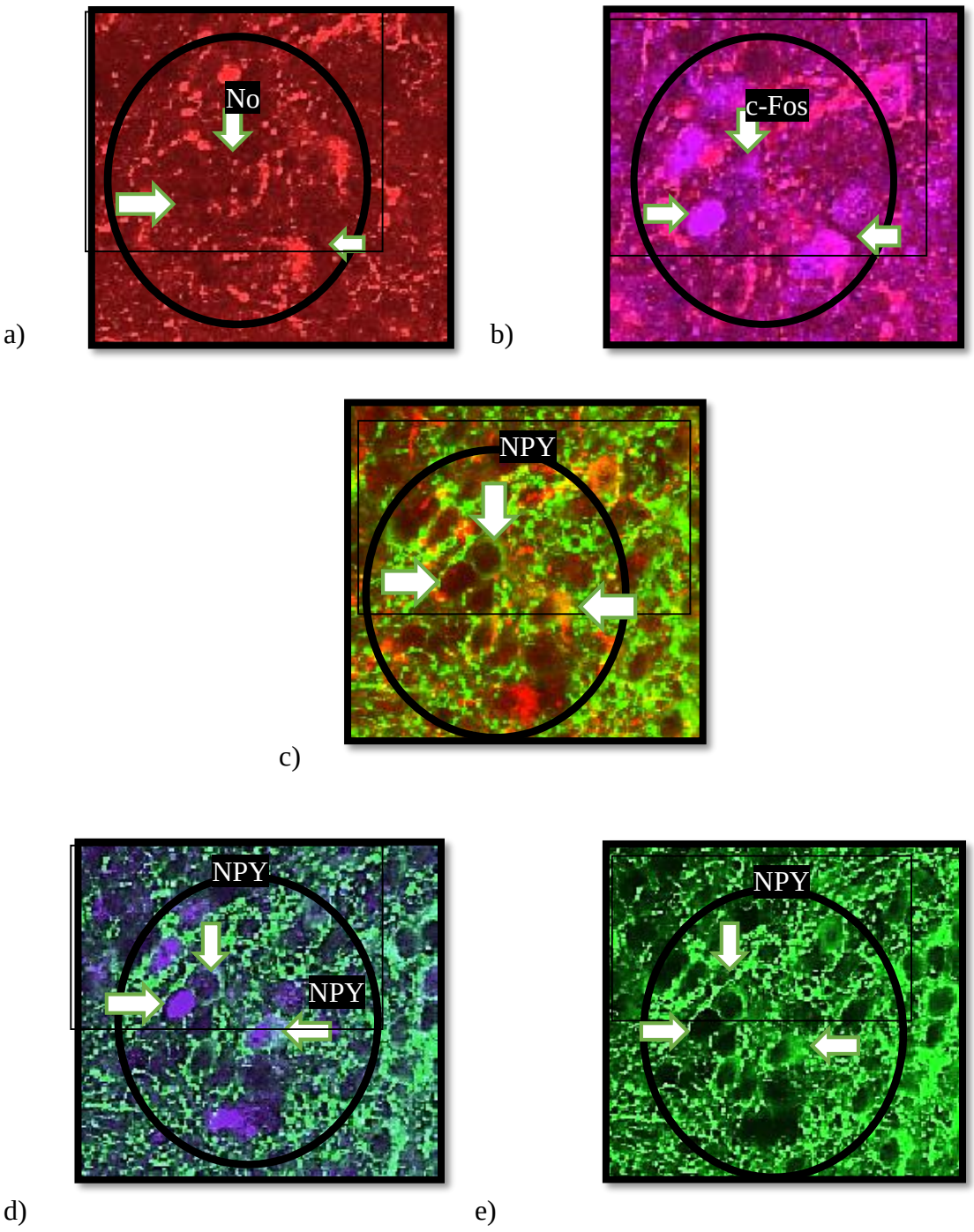
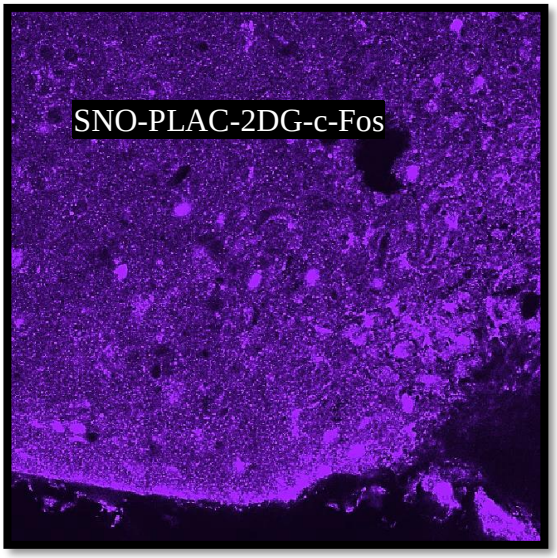
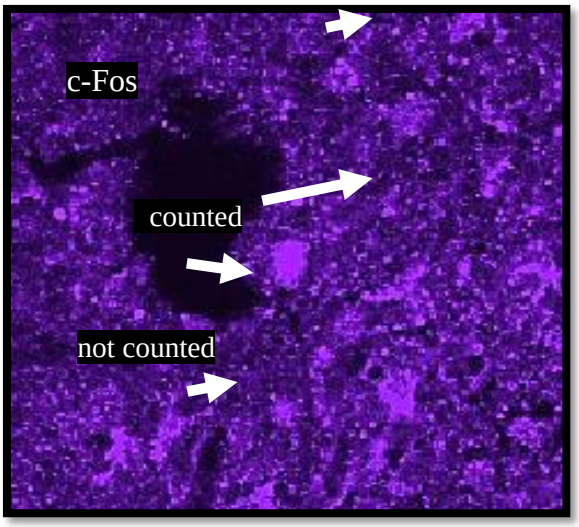


Figure 24 Immunohistochemistry Cell Counting

Activity example for cell co-localization: Colour image c-Fos (purple):- Fos-like early gene expression; NPY (green): Neuropeptide -Y & α-MSH (red) Alpha-Melanocyte-stimulating hormone. c-Fos mean 88.2± 19.18, in brain slices from the ARC: - arcuate nucleus of the hypothalamus in SNO (n=5) representative of *Snord116del* mice, ingesting chronic treatment 100CFE: - *Caralluma fimbriata* extract, at 100mg/kg/d, with appetite signalling reagents, 2DG: - 2-deoxy-glucose.



a/

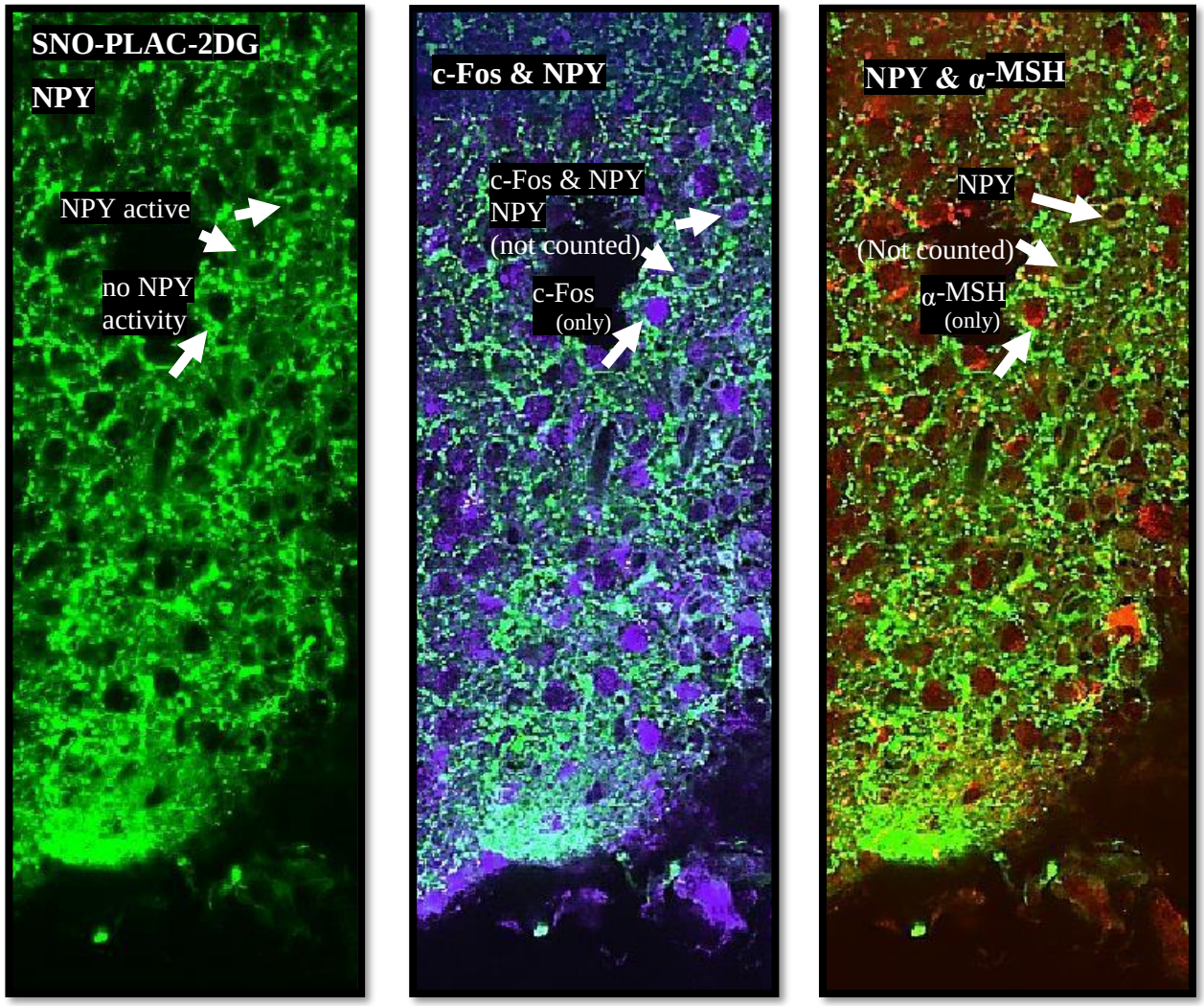


b/

Figure 25 Cell c-Fos Activity in the Hypothalamus

Example of choice for Immunohistochemistry counting of c-Fos:- Fos-like early gene expression in a brain slice from the ARC: - arcuate nucleus of the hypothalamus, SNO:-*Snord116del* mouse model (n=5), ingesting the PLAC: – placebo of maltodextrin/cabbage leaf ,when administered the appetite signalling reagent 2DG: - 2-deoxy-glucose at 90-120 mins before perfusion: a/ brain slice representative with mean c-Fos 49.6 ± 16.68 b/ close up of c-Fos activity.

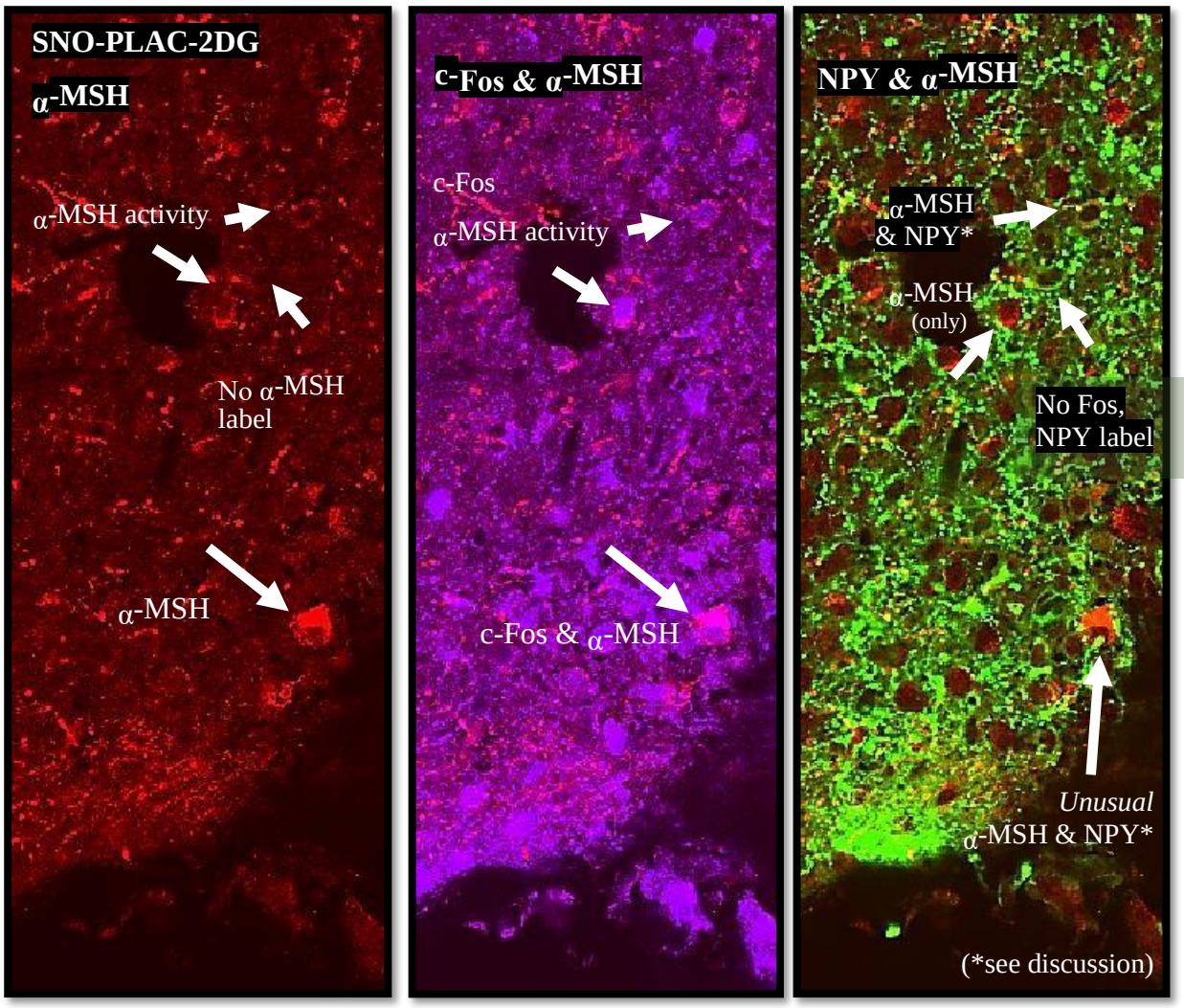
Note: This representative image has been chosen for instructive purposes due to the distinct visual break in the slice which clearly anchors the cell positioning. The arrows establish the inclusion of active cells in this slide and the images on the next page.



a/ b/ c/

Figure 26 Neurotransmitter co-localization One

Immunohistochemistry c-Fos (purple):- Fos-like early gene expression in a brain slice from the ARC: - arcuate nucleus of the hypothalamus: mean 49.6 ± 16.68 in SNO:-*Snord116del* (n=5) ingesting the PLAC: – placebo of maltodextrin/cabbage leaf ,when administered the appetite signalling reagent 2DG: - 2-deoxyglucose at 90-120 mins before perfusion. a) NPY (green): neuropeptide–Y, b) NPY and c-Fos: mean 21 ± 15.93, c) α-MSH (red): alpha - Melanocyte-stimulating hormone and c-Fos.



a/ b/ c/

Figure 27 Secondary Antibody Co-localization

Immunohistochemistry c-Fos:- Fos-like early gene expression in a brain slice from the ARC: - arcuate nucleus of the hypothalamus, mean 49.6 ± 16.68 in the SNO:-*Snord116del* mouse model (n=5), ingesting the PLAC: – placebo of maltodextrin/cabbage leaf ,when administered the appetite signalling reagent 2DG: - 2-deoxy-glucose at 90-120 mins before perfusion. a) α-MSH: alpha - Melanocyte-stimulating hormone b) α-MSH & c-Fos: mean 6.8 ± 12.11 c) NPY: neuropeptide–Y and c-Fos.

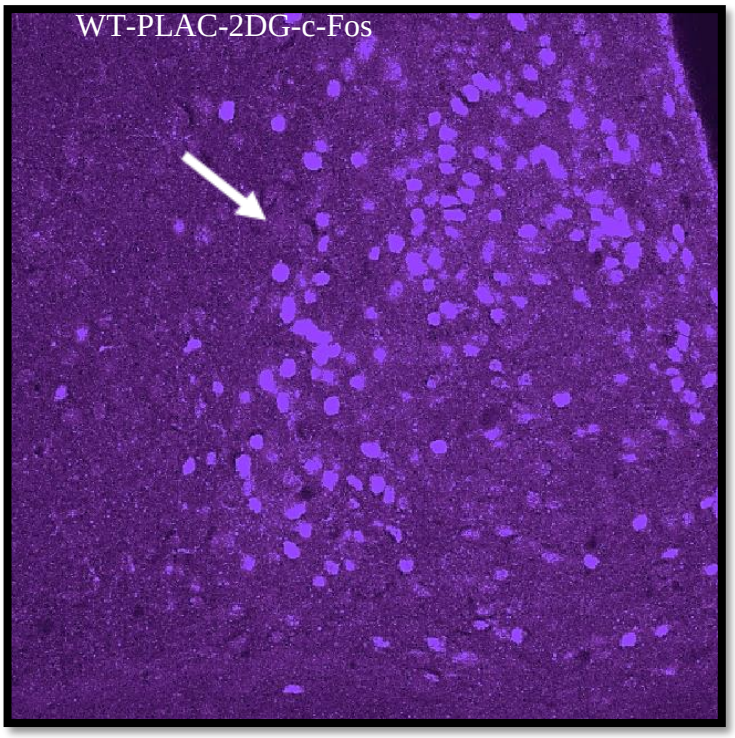


Figure 28 Mouse Model c-Fos activity

Immunohistochemistry presenting strongest c-Fos (purple):- Fos-like early gene expression in a brain slice from the ARC: - arcuate nucleus of the hypothalamus, mean 218.4 ± 55.38, (*P* <0.001**) in WT: - C57BL/6 wild type, (n=5) per group, ingesting PLAC: – placebo of maltodextrin/cabbage leaf, when administered the appetite signalling reagent 2DG: - 2-deoxy-glucose at 90-120 mins before perfusion.

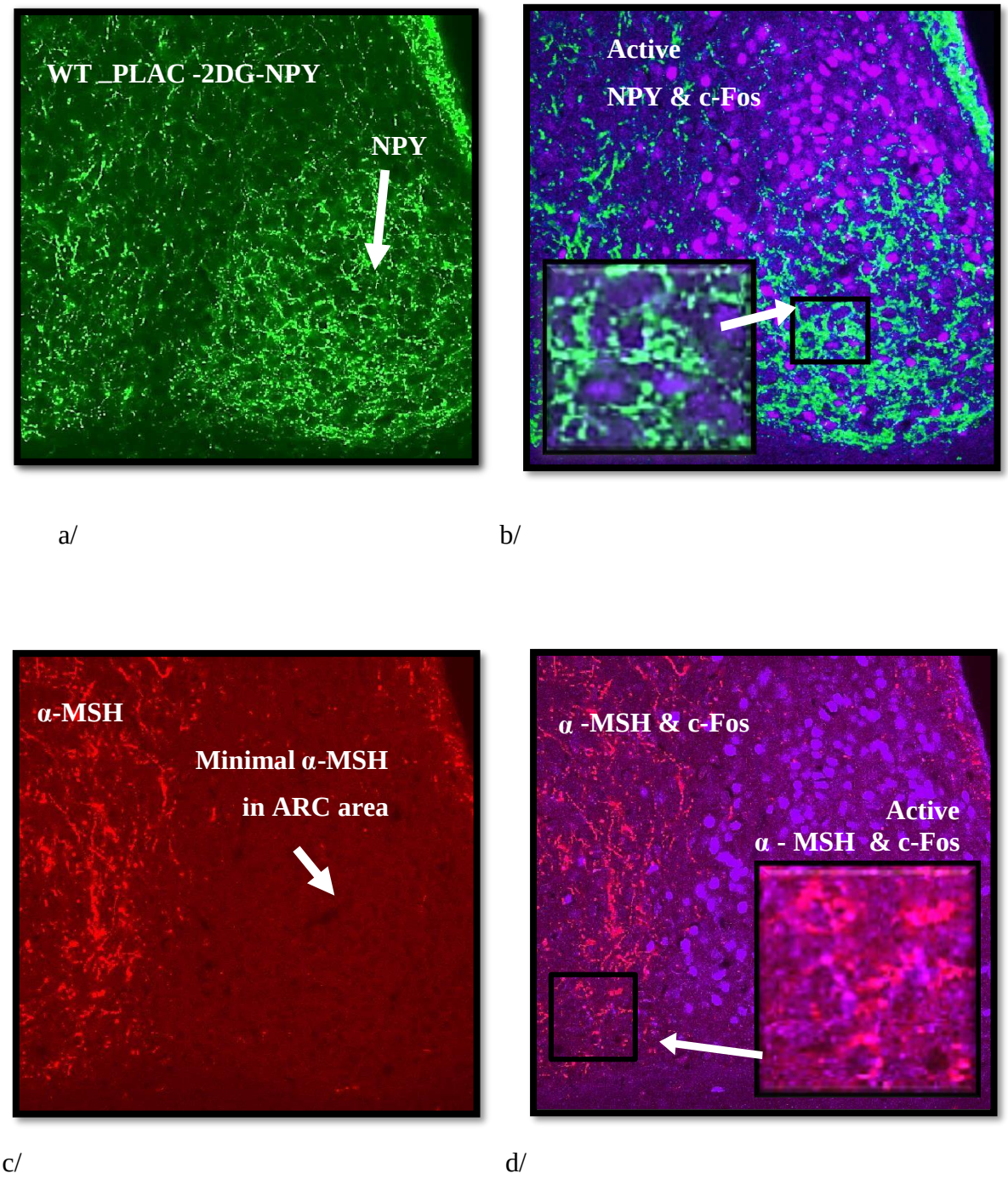


Figure 29 Neurotransmitter Activity in the Arcuate Nucleus of the Hypothalamus

Immunohistochemistry of WT: - C57BL/6 wild type, (n=5) per group, ingesting PLAC: – placebo of maltodextrin/cabbage leaf, when administered the appetite signalling reagent 2DG: - 2-deoxy-glucose at 90-120 mins before perfusion. This is the highest activity of c-Fos (purple):- Fos-like early gene expression in a brain slice from the ARC: - arcuate nucleus of the hypothalamus, mean 218.4 ± 55.38, (*P* <0.001**): a/ NPY (green): neuropeptide–Y, b/ NPY and c-Fos mean 159.8 ± 65.53 (*P* <0.001**) c/ α-MSH (red): alpha - Melanocyte-stimulating hormone and d/ α-MSH and c-Fos mean 20.8 ± 11.88.

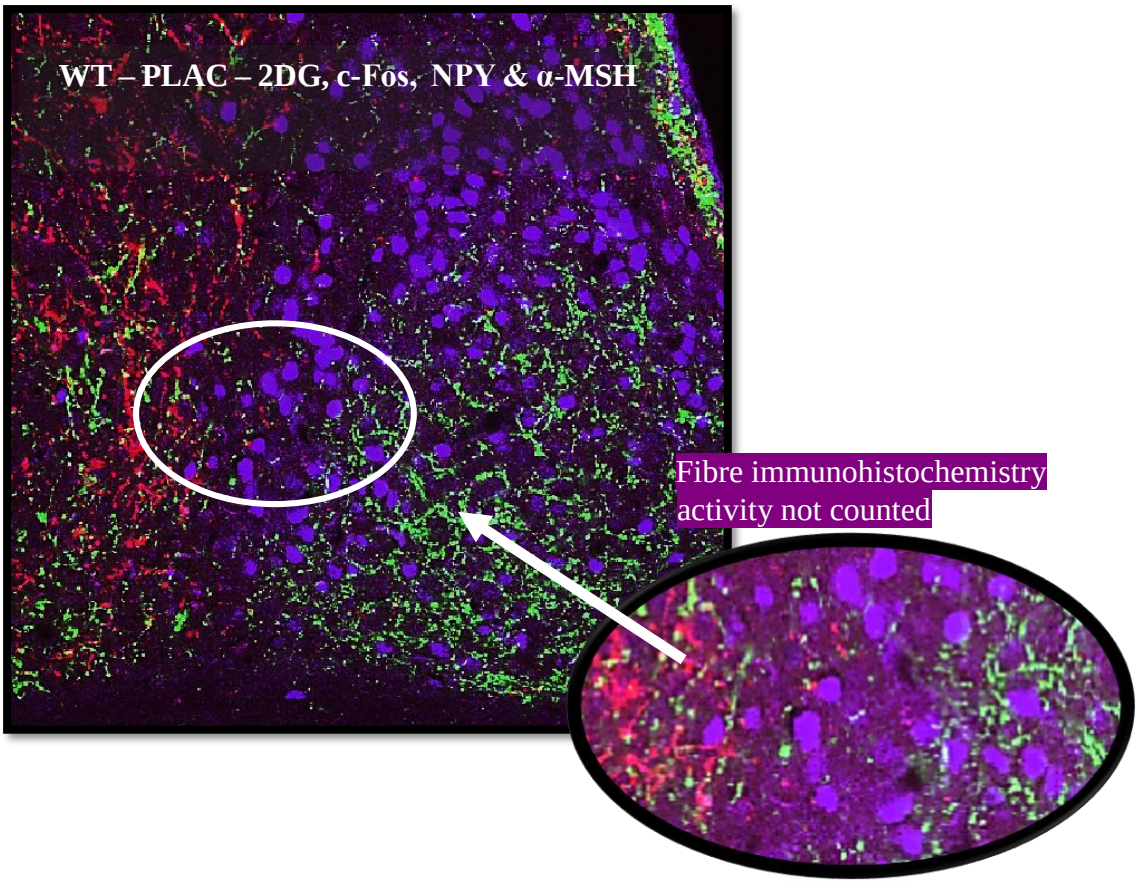


Figure 30 Staining of Fibrous Tissue

Immunohistochemistry WT: - C57BL/6 wild type, mouse (n=5) per group, ingesting PLAC: – placebo of maltodextrin/cabbage leaf, when administered the appetite signalling reagent 2DG: - 2-deoxy-glucose at 90-120 mins before perfusion. This is the highest activity of c-Fos (purple):- Fos-like early gene expression in a brain slice from the ARC: - arcuate nucleus of the hypothalamus, mean 218.4 ± 55.38, (*P* <0.001**): a) NPY (green): neuropeptide–Y, α-MSH (red): alpha - Melanocyte-stimulating hormone.

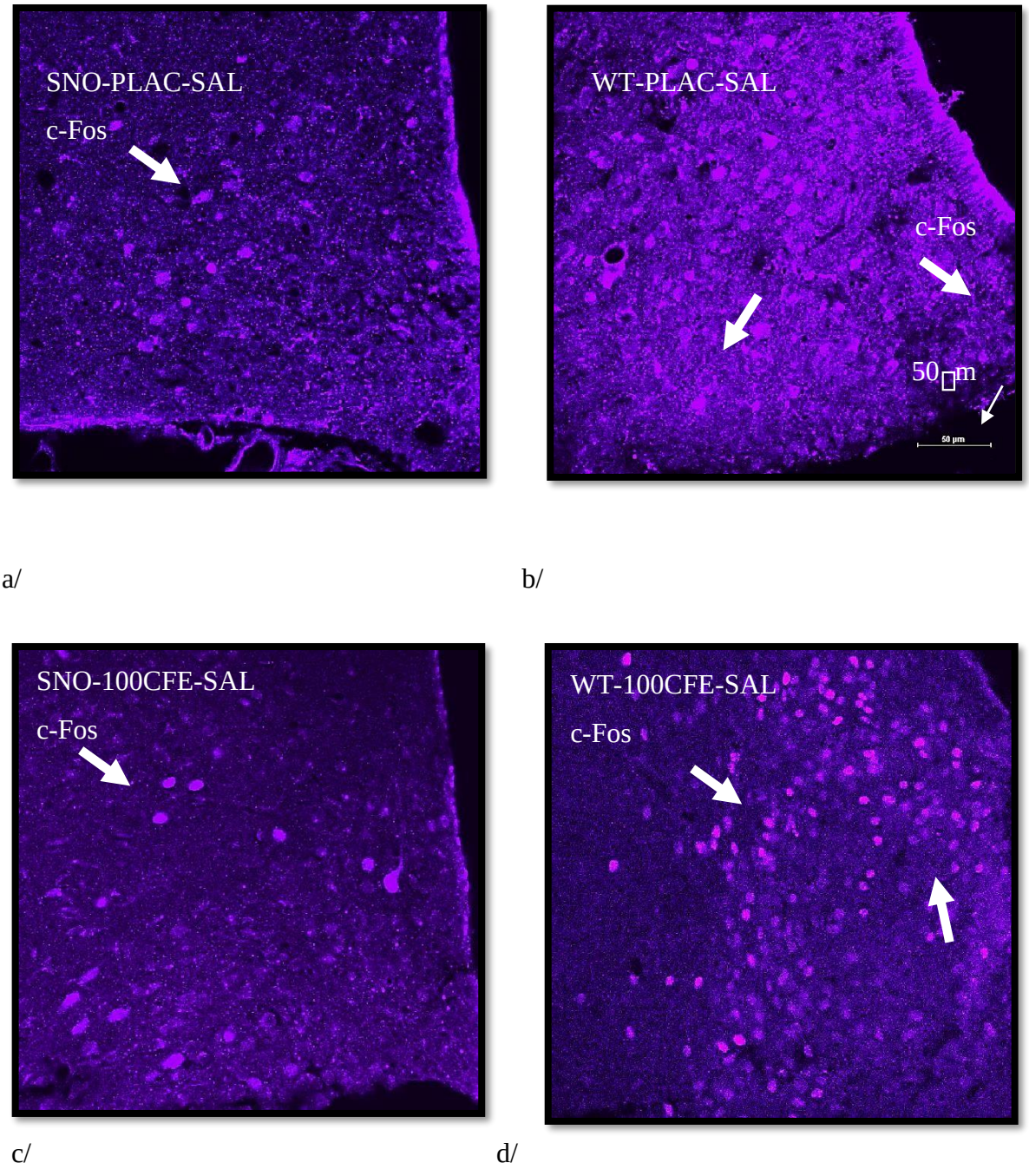


Figure 31 Group comparisons of Arcuate Nucleus c-Fos Activity

Figures Immunohistochemistry c-Fos (purple):- Fos-like early gene expression in a brain slice from the ARC: - arcuate nucleus of the hypothalamus from either the SNO:-*Snord116del* mouse model (n=5), or WT- C57BL/6 wild type, mouse (n=5) per group, ingesting chronic treatment of the PLAC: – placebo of maltodextrin/cabbage leaf, or 100CFE: - *Caralluma fimbriata* extract, at 100mg/kg/d, when injected with SAL –saline at 90 mins before perfusion a/SNO-PLAC 51 ± 15.84; b/ WT-PLAC 126.60 ± 40.12 (*P*=0.02*), c/ SNO-100CFE, 118.8 ± 28.41 d/ WT-100CFE 138.60 ± 24.13; SNO-PLAC 51 ±15.84 (*P*=0.006**).

Table 19 Paraventricular Cell Count Comparisons

Pairwise comparisons of average counts of cells for florescent c-Fos, NPY and α-MSH activity in the paraventricular nucleus of the Snord116 deletion and WT control mouse brain sections.

PVN	c-Fos	Pairwise comparison		
c-Fos	**Mean**	**SD**		***P value***
SNO-100CFE -2DG	78	± 16.29	All groups	(NS)
SNO-100CFE-SAL	79.6	± 29.19	All groups	(NS)
SNO-PLAC-2DG	51	± 15.84	WT-PLAC-2DG	0.005**
SNO-PLAC-SAL	97.2	± 43.05	All groups	(NS)
WT-100CFE-2DG	97.4	± 39.03	All groups	(NS)
WT-100CFE-SAL	102.8	± 27.87	All groups	(NS)
			SNO-100CFE -2DG	0.17 (NS)
			SNO-100CFE-SAL	0.22 (NS)
WT-PLAC-2DG	138.2	± 49.17	SNO-PLAC-2DG	0.005**
WT-PLAC-SAL	107.8	± 23.49	All groups	(NS)
NPY				(NS)
SNO-100CFE -2DG	37.6	± 23.73	All groups	(NS)
SNO-100CFE-SAL	53.8	± 28.08	All groups	(NS)
SNO-PLAC-2DG	24	± 12.94	WT-PLAC-2DG	0.03*
SNO-PLAC-SAL	42.8	± 27.66	All groups	(NS)
WT-100CFE-2DG	63.2	± 27.6	SNO-PLAC-2DG	0.03*(NS)
WT-100CFE-SAL	54.6	± 13.87	All groups	(NS)
WT-PLAC-2DG	76	± 18.07	SNO-PLAC-2DG	0.03*
WT-PLAC-SAL	48.4	± 24.47	All groups	(NS)
α-MSH				(NS)
SNO-100CFE -2DG	6.4	± 8.9	All groups	(NS)
SNO-100CFE-SAL	9.2	± 7.05	All groups	(NS)
SNO-PLAC-2DG	3.6	± 3.21	All groups	(NS)
SNO-PLAC-SAL	1.75	± 2.19	All groups	(NS)
WT-100CFE-2DG	2.6	± 4.16	All groups	(NS)
WT-100CFE-SAL	5	± 3.87	All groups	(NS)
WT-PLAC-2DG	7.8	± 6.42	All groups	(NS)
WT-PLAC-SAL	3.6	± 3.13	All groups	(NS)

Table 19. ANOVA Post Hoc pairwise comparisons with estimated marginal means and SD - standard deviation. Results of immunohistochemistry cell counts for c-Fos:- Fos-like early gene expression, NPY: neuropeptide–Y and α-MSH: alpha - Melanocyte-stimulating hormone, in brain slices from the PVN: - paraventricular nucleus of the hypothalamus of two mouse strains: SNO: - the Garvan *Snord116del* (n=5) and WT: - C57BL/6 wild type, (n=5) per group. Animals were ingesting either chronic treatment 100CFE: - *Caralluma fimbriata* extract, at 100mg/kg/d or PLAC: – placebo of maltodextrin/cabbage leaf with appetite signalling reagents, 2DG: - 2-deoxy-glucose compared to the control of SAL: - saline.

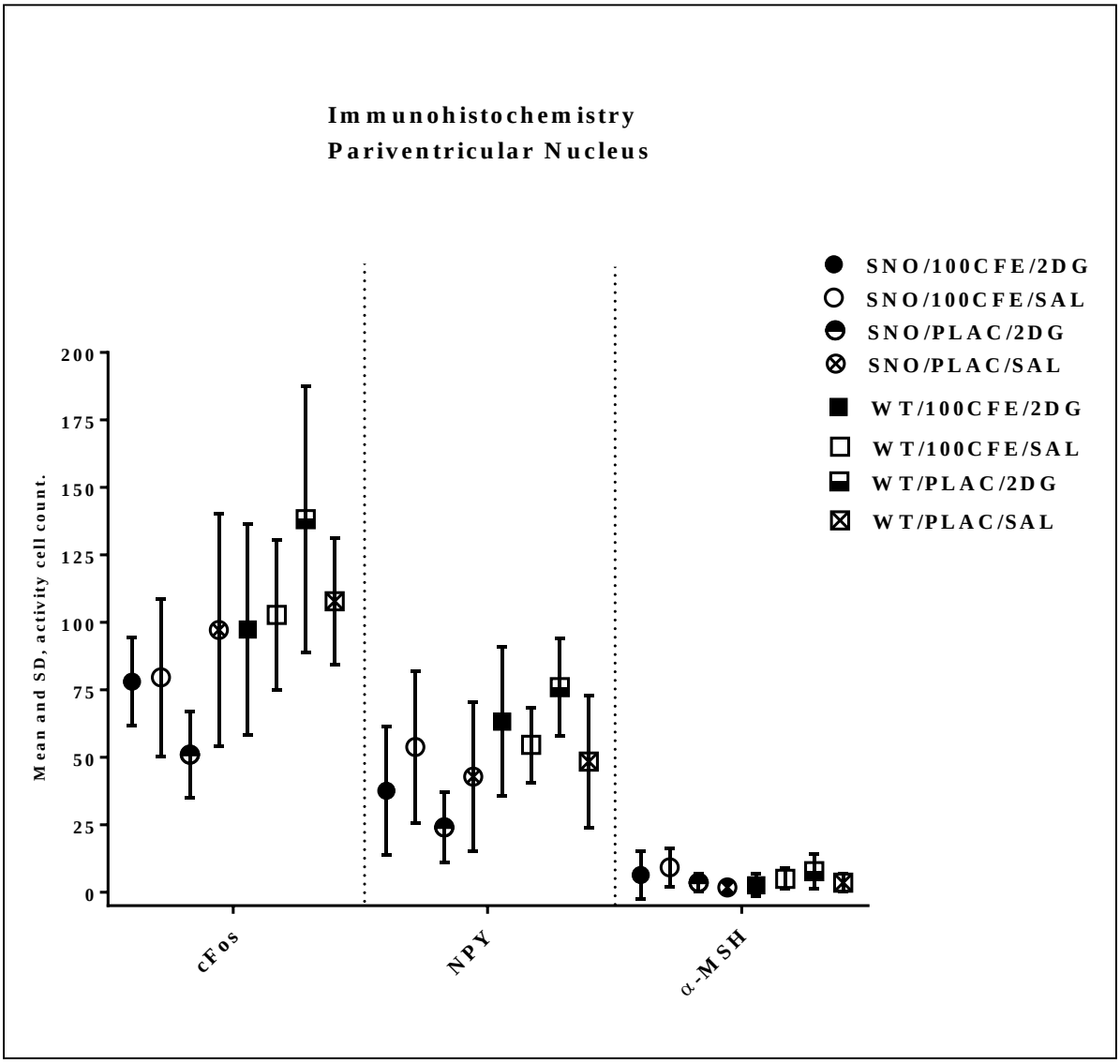


Figure 32 Paraventricular Comparisons of Treatment and Strains

Graph of estimated marginal means and SD - standard deviation for immunohistochemistry cell counts of c-Fos:- Fos-like early gene expression, NPY: neuropeptide–Y and α-MSH: alpha – Melanocyte stimulating hormone, in brain slices from the PVN: - paraventricular nucleus of the hypothalamus of two mouse strains: SNO: - Garvan *Snord116de*l mouse model (n=5) and WT: - C57BL/6 wild type, (n=5) per group. Animals were ingesting either chronic treatment 100CFE: - Caralluma fimbriata extract, at 100mg/kg/d or PLAC: – placebo of maltodextrin/cabbage leaf with appetite signalling reagents, 2DG: - 2-deoxy-glucose, compared to the control of SAL: - saline.

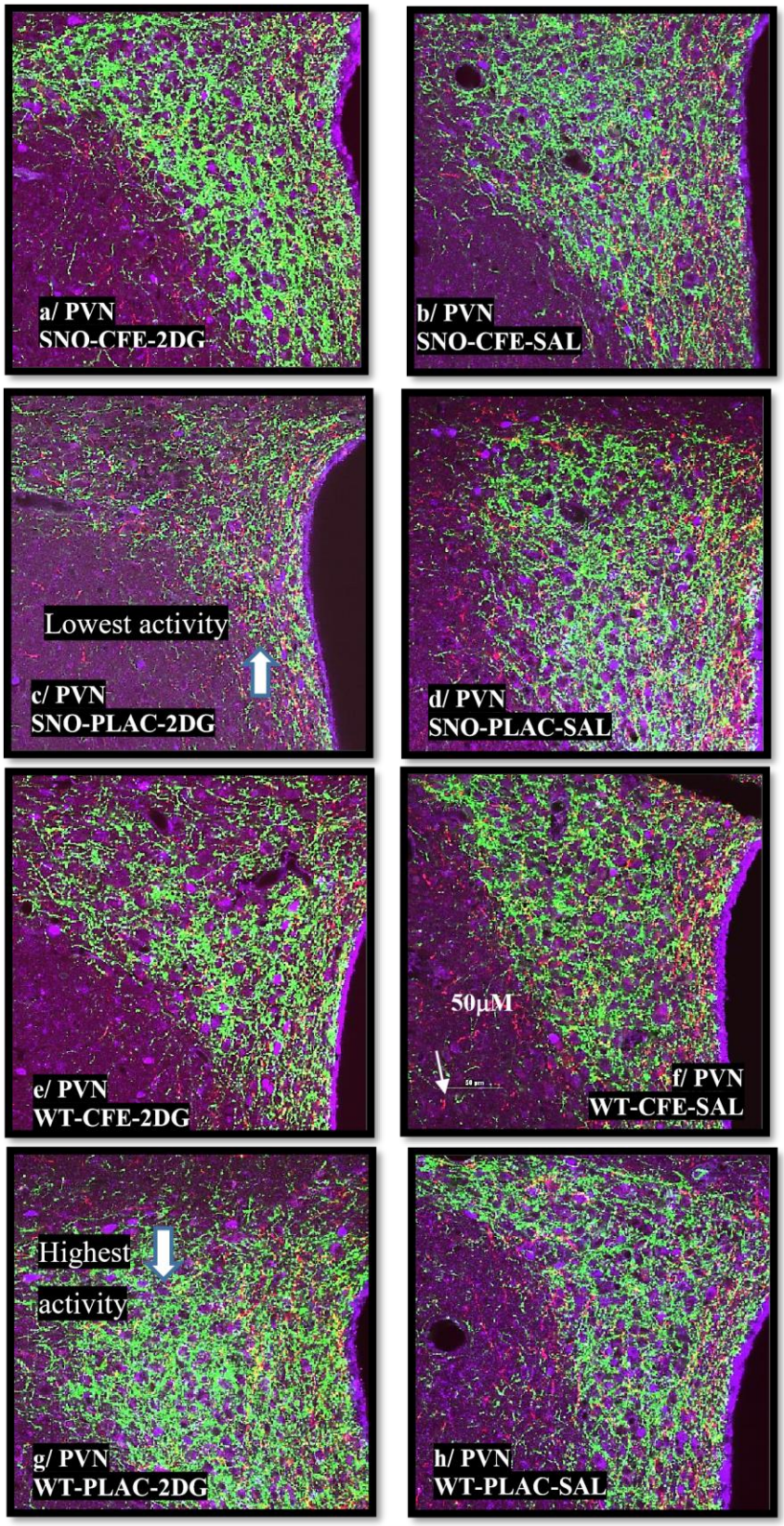


Figure 33 Immunohistochemistry c-Fos:- Fos-like early gene expression, NPY: neuropeptide–Y and α-MSH: alpha - Melanocyte-stimulating hormone in brain slices from the PVN: - paraventricular nucleus of the hypothalamus, in SNO: - the Garvan *Snord116del* mouse model (n=5) per group and WT: - C57BL/6 wild type, (n=5) per group. Animals were ingesting either chronic treatment 100CFE: - *Caralluma fimbriata* extract, at 100mg/kg/d or PLAC: – placebo of maltodextrin/cabbage leaf with appetite signalling reagents, 2DG: - 2-deoxy-glucose compared to the control of SAL: - saline. Results 90 minutes after 2DG, c-Fos c/SNO-PLAC 51 ± 15.84; WT-PLAC 138.2 ± 49.17 (P = 0.005 **), NPY: - SNO-PLAC 24 ± 12.94; WT-PLAC, 76 ± 18.07 (P = 0.03 *), α-MSH not significant.

Figure 33 Group Cell Activity in the Paraventricular
c-Fos (purple):-c-Fos; NPY (green): neuropeptide–Y & α-MSH (red): alpha - Melanocyte-stimulating hormone.

The images of the PVN obviously present the higher appetite in both c-Fos activity and NPY activity in the WT-PLAC groups both stimulated figures 33 g/ and control h/. These PVN images also present the lower appetite of the SNO-PLAC group in figure 33 c/ when stimulated by 2DG in the PVN. The results are as per table 2DG c-Fos: WT-PLAC138.2 ± 49.17; SNOPLAC 51 ± 15.84 (P = 0.005), also NPY WT-PLAC76 ± 18.07; SNO-PLAC 24 ± 12.94 (P = 0.03), the α-MSH was not significant.

Though the images of the SNO-100CFE stimulated by 2DG looks strongly active in NPY, much of this activity was fibrous tissue, seen clearly in figure 30. These results were actually lower than the saline (NS), PVN 2DG: SNO-100CFE 37.6 ± 23.78; SAL: SNO-PLAC 53.8 ± 28.08 (NS). Once again the higher SD was apparent. The last images (Figure 33) demonstrate the minimal activity of cFos and NPY in the PVN of the SNO animals. Figure 34 also presents CFos activity in the PVN, with less c-Fos activity in the SNO strain

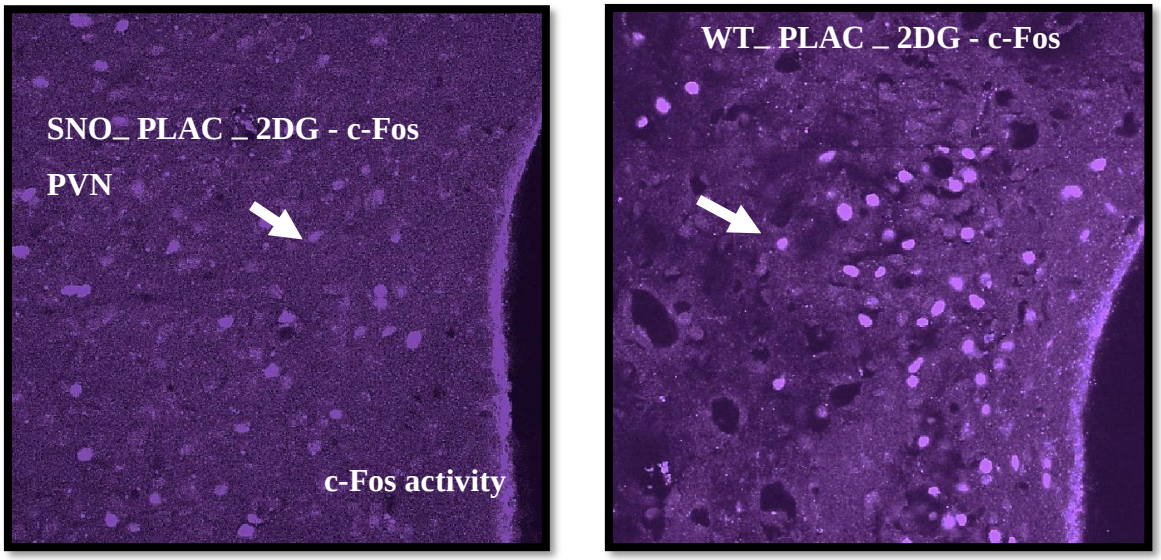


Figure 34 C-Fos activity Paraventricular Mouse Strain Comparisons

Figure Immunohistochemistry c-Fos (purple):- Fos-like early gene expression in a brain slice from the PVN: - paraventricular nucleus of the hypothalamus, SNO: - the Garvan *Snord116del* mouse model (n=5) per group, and WT- wild type (n=5), ingesting PLAC: – placebo of maltodextrin/cabbage leaf, when administered the appetite signalling reagent 2DG: - 2-deoxy-glucose at 90-120 mins before perfusionFos:- SNO-PLAC-2DG 51 ± 15.84; WT-PLAC-2DG138 ± 49.17, (p=0.005**).

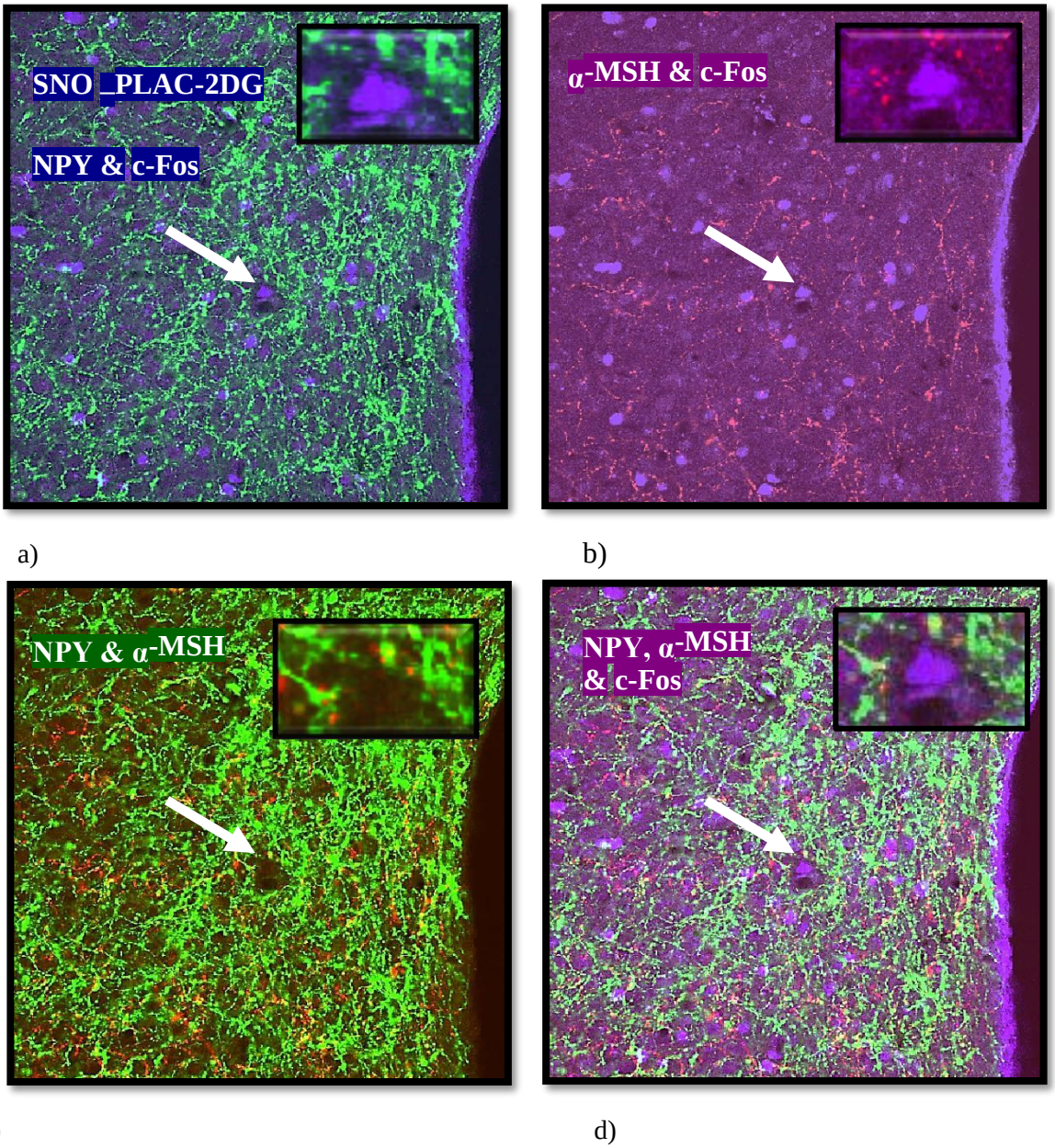


Figure 35 PVN C-Fos, NPY and α_MSH Activity Labelling

Immunohistochemistry c-Fos (purple):- Fos-like early gene expression in a brain slice from the PVN: - paraventricular nucleus of the hypothalamus, in SNO: - the Garvan Snord116del mouse model (n=5) per group, ingesting PLAC: – placebo of maltodextrin/cabbage leaf, when administered the appetite signalling reagent 2DG: - 2-deoxy-glucose at 90-120 mins before perfusion. a) c-Fos & NPY (green): neuropeptide–Y:-SNO-PLAC-2DG 24 ± 12.94; WT-PLAC-2DG, 76 ± 18.07, (P=0.03*), b) α-MSH (red): alpha - Melanocyte-stimulating hormone and c-Fos mean c) α-MSH and c-Fos, not significant and all together with insets of c-Fos activity (only).

6.4.2 Water appetite activity

The images of the MnPo immunohistochemistry (Figures 39 and 40), for water appetite at the time of the perfusion presented very similar activity to the behavioural studies. These confirmed the SNO-PLAC animals to be less thirsty as in the behavioural studies where they drank the least amount of water. In the immunohistochemistry results for the MnPO (Table 20 and Figure 38) in regards to anticipatory water appetite – at the time of the perfusion, the results were also diminished in the stimulated SNO-PLAC and were again strongest in the WT-PLAC, due to increased NPY activity, MnPO 2DG: - c-Fos, SNO-PLAC 63.8 ± 17.38; WT-PLAC 84.8 ± 27.34 (*P*= 0.01*), NPY, SNO-PLAC 12.4 ± 5.22; WT-PLAC 63.4± 37.54 (*P*= 0.03*). The graph on page # also clearly presents the drop in thirst in the SNO-SAL animal. Within the table though not all significant trends also been highlighted.

Further looking at the images mapping neuronal activity in the animals administered MA (Figures 41 & 42) on pages 248 and 249, the SNO animals figure a- d/ demonstrated c-Fos activity with a large amount of fibrous NPY, without co-labelling the Fos activity. In the SNOPLAC-MnPO stimulated by MA there was very little c-Fos activity, which seemed to follow the behavioural SNO results in food and water appetite. On the other hand, during MA stimulation, WT brain AIO had very high c-Fos activity figure e/- h/ and less interaction with NPY. Why there was minimal NPY activity is less obvious as the c-Fos activity was highly active with the strongest expression in the PVN area. More study will need to be conducted to ascertain a reason for this mediation in signalling.

Table 20 Comparisons of Strain and Treatment in the Median Preoptic

Pairwise comparisons of average counts of cells for florescent c-Fos, NPY and α-MSH activity in the median preoptic nucleus of the Snord116 deletion and WT control mouse brain sections.

MnPO	c-Fos		Pairwise comparison	
c-Fos	**Mean**	**SD**		***P value***
SNO-100CFE -2DG	73	± 28.43	SNO-PLAC-2DG	0.09 (NS)
SNO-100CFE-SAL	84.2	± 15.02	SNO-PLAC-2DG	0.01*
			WT-100CFE-SAL	<0.001**
			WT-PLAC-2DG	0.01*
SNO-PLAC-2DG	22	± 3.81	WT-PLAC-SAL	<0.001**
SNO-PLAC-SAL	63.8	± 17.38	WT-PLAC-SAL	0.16 (NS)
			WT-100CFE-SAL	0.01*
WT-100CFE-2DG	48.4	± 15.47	WT-PLAC-SAL	0.01*
WT-100CFE-SAL	110.4	± 42.82	All noted above	To <0.001**
WT-PLAC-2DG	84.8	± 27.34	SNO-PLAC-2DG	0.01*
WT-PLAC-SAL	110.8	± 29.35	All noted above	To - <0.001**
NPY				(NS)
SNO-100CFE -2DG	21	± 22.8	All groups	(NS)
SNO-100CFE-SAL	47.2	± 16.02	All groups	(NS)
SNO-PLAC-2DG	12.4	± 5.22	WT-PLAC-2DG	0.03*
SNO-PLAC-SAL	35.6	± 10.38	All groups	(NS)
WT-100CFE-2DG	34.8	± 15.16	All groups	(NS)
WT-100CFE-SAL	35.8	± 30.11	All groups	(NS)
WT-PLAC-2DG	63.4	± 37.54	SNO-PLAC-2DG	0.03*
WT-PLAC-SAL	51	± 33.33	All groups	(NS)
α-MSH				(NS)
SNO-100CFE -2DG	3.6	± 4.15	All groups	(NS)
SNO-100CFE-SAL	7.4	± 7.13	All groups	(NS)
SNO-PLAC-2DG	0.8	± 1.3	All groups	(NS)
SNO-PLAC-SAL	0.8	± 0.89	All groups	(NS)
WT-100CFE-2DG	0.4	± 0.89	All groups	(NS)
WT-100CFE-SAL	0.8	± 1.09	All groups	(NS)
WT-PLAC-2DG	3.2	± 4.08	All groups	(NS)
WT-PLAC-SAL	9.8	± 11.71	All groups	(NS)

Table 20. ANOVA Post Hoc pairwise comparisons with estimated marginal means and SD - standard deviation. Results of immunohistochemistry cell counts for c-Fos:- Fos-like early gene expression, NPY: neuropeptide–Y and α-MSH: alpha - Melanocyte-stimulating hormone, in brain slices from the MnPO: - median preoptic nucleus of the lamina terminalis of two mouse strains: SNO: - Garvan *Snord116del* (n=5) and WT: - C57BL/6 wild type, (n=5) per group. Animals were ingesting either chronic treatment 100CFE: - *Caralluma fimbriata* extract, at 100mg/kg/d or PLAC: – placebo of maltodextrin/cabbage leaf with appetite signalling reagents, 2DG: - 2-deoxy-glucose compared to the control of SAL: - saline

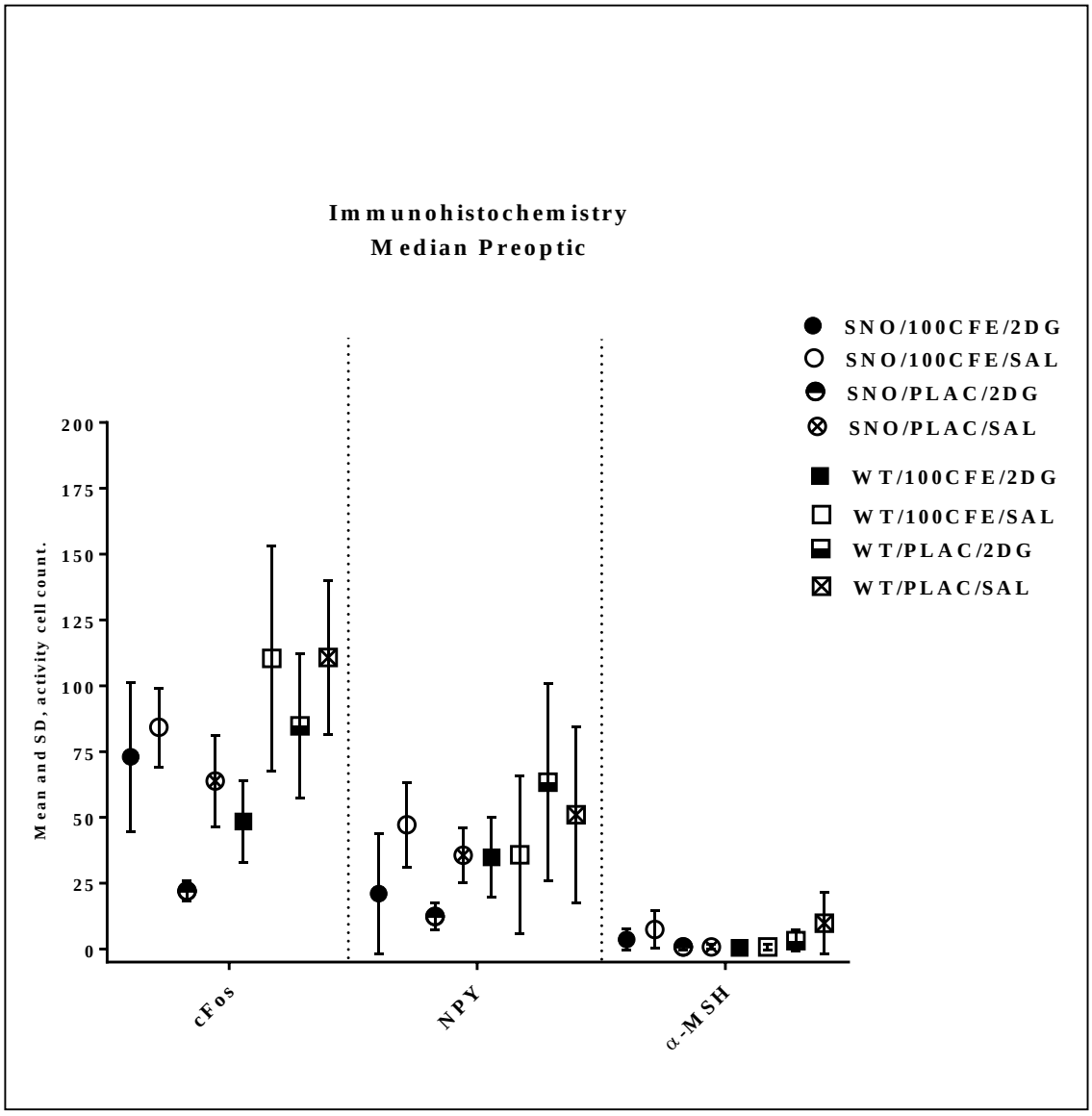


Figure 36 Median Preoptic Comparisons of Group Cell Co-Localization

Graph of estimated marginal means and SD - standard deviation for immunohistochemistry cell counts of c-Fos:- Fos-like early gene expression, NPY: neuropeptide–Y and α-MSH: alpha - Melanocyte-stimulating hormone, in brain slices from the MnPO: - median preoptic nucleus of the lamina terminalis of two mouse strains: SNO: - Garvan Snord116del (n=5) and WT: - C57BL/6 wild type, (n=5) per group. Animals were ingesting either chronic treatment 100CFE: - Caralluma fimbriata extract, at 100mg/kg/d or PLAC: – placebo of maltodextrin/cabbage leaf with appetite signalling reagents, 2DG: - 2-deoxy-glucose compared to the control of SAL: - saline

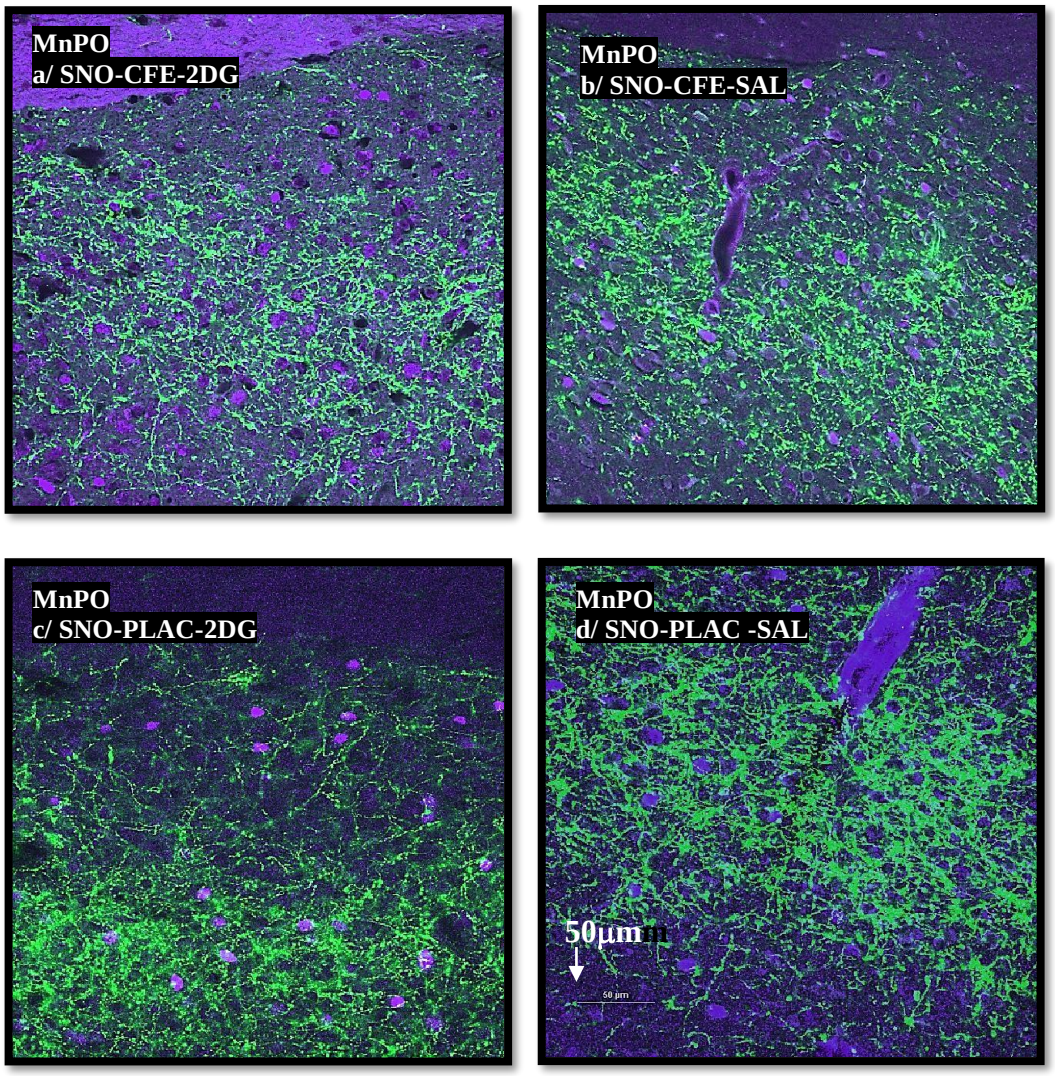


Figure 37 Median Preoptic c-Fos and Neuropeptide-Y in the *Snord116* Strain

Two pages of comparisons: a/ - h/ Immunohistochemistry c-Fos (purple :- Fos-like early gene expression and NPY (green): neuropeptide–Y, in brain slices from the MnPO:- Median Preoptic Nucleus in SNO: - the Garvan Snord116del mouse model and WT: - C57BL/6 wild type, (n=5) per group. Animals were ingesting either CFE: - Caralluma fimbriata extract, at 100mg/kg/d with the appetite signalling reagents, 2DG: - 2-deoxy-glucose, compared to the control of SAL: - saline, c-Fos results:- b/SNO-100CFE 84.2 ± 15.02; to c/ SNO-PLAC-2DG 22 ± 3.81, (*P*=0.01*); b/ to f/ WT-100CFE-SAL 110 ± 29.35, (*P*= <0.001**); b/to g/ WT-PLAC-2DG 84.80 ± 27.34,(*P*=0.01*); c/ to g/ (*P*= <0.001**); d/ WT-100CFE-2DG 48.4 ± 15.47; to h/ WT-PLAC-SAL 110.80 ± 29.35, (*P*=0.01*), g/ to c/,(*P*=0.01*).

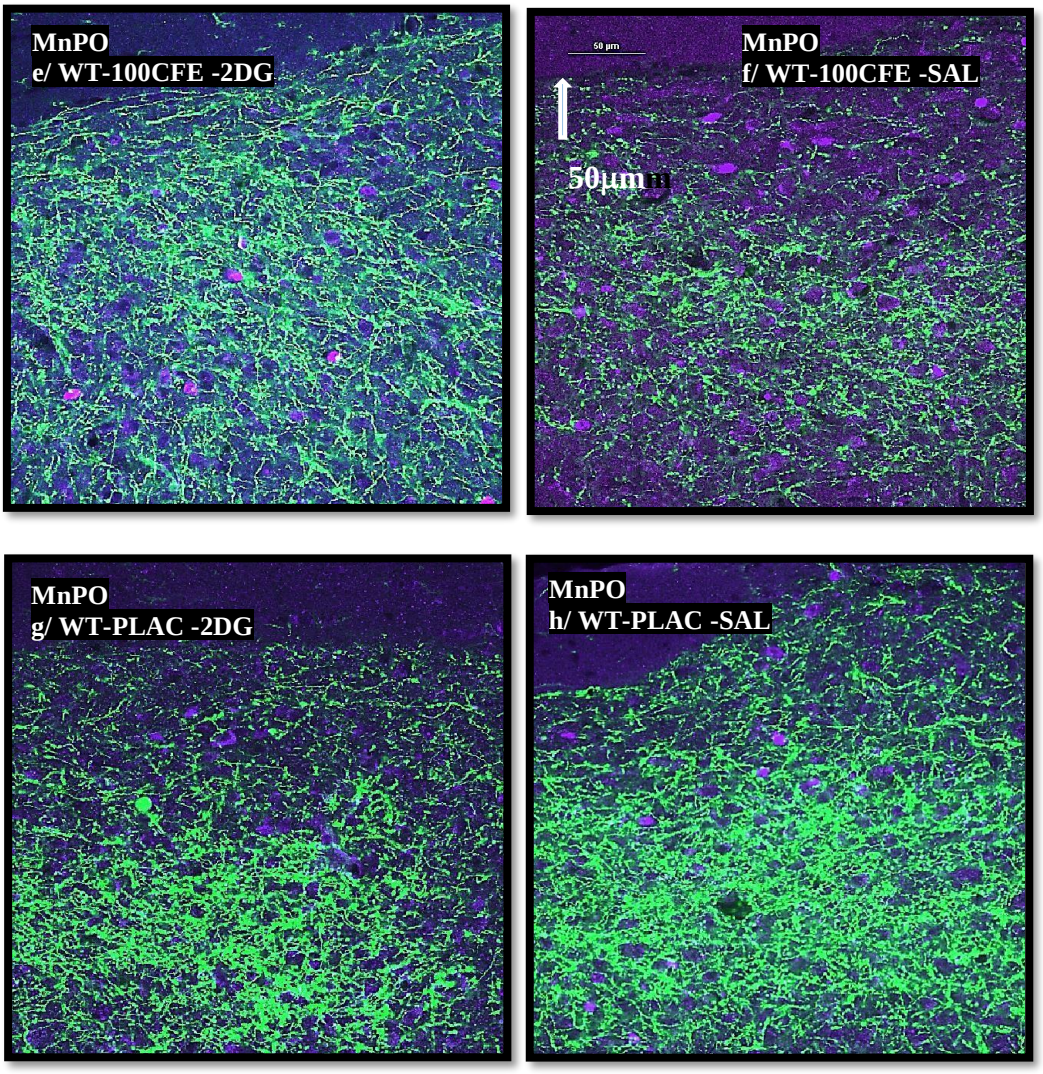
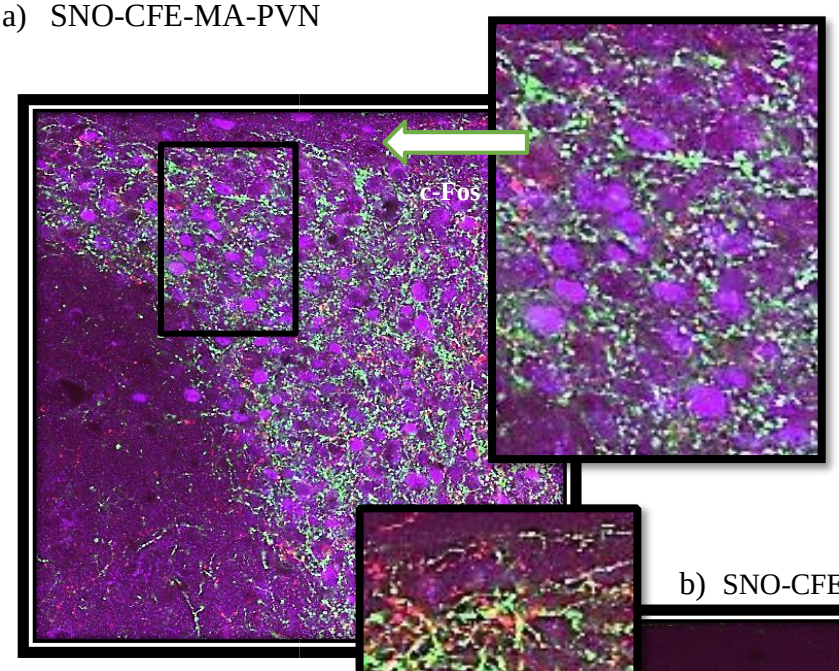


Figure 38 Median Preoptic c-Fos and Neuropeptide-Y in the Wild Type Strain

Two pages of comparisons a/ - h/ Immunohistochemistry c-Fos (purple) and NPY (green): neuropeptide–Y, in brain slices from the MnPO:- Median Preoptic Nucleus in SNO: - the Garvan *Snord116del* mouse model and WT: - C57BL/6 wild type, (n=5) per group. Animals were ingesting the PLAC: – placebo of maltodextrin/cabbage leaf with the appetite signalling reagents, 2DG: - 2-deoxy-glucose compared to the control of SAL: - saline. NPY:- c/ SNO-PLAC-2DG 12.40 ± 5.22; to g/ WT-PLAC-2DG 63.4 ± 37.54, (P = 0.03*)

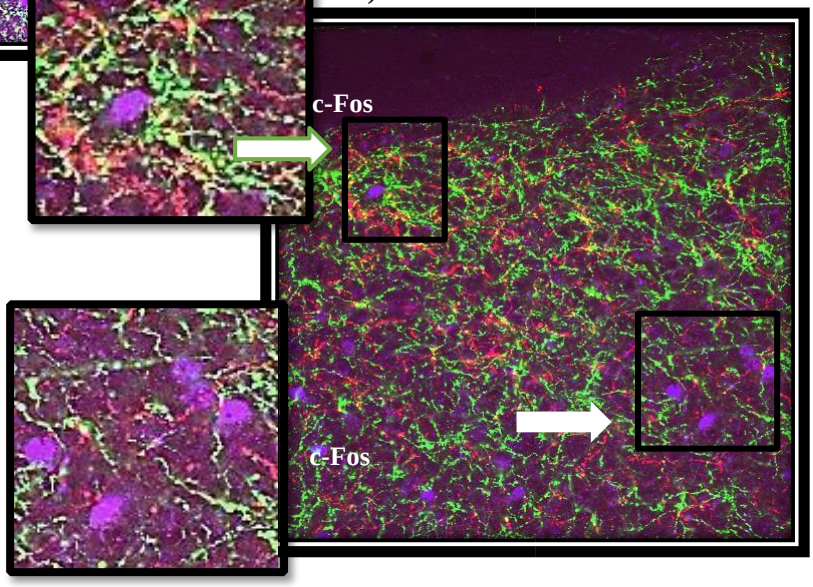
During earlier investigations chapter five found some unusual fluid intake in regards to CFE's reduction in appetite and deprivation in the SNO animals. Therefore, the MnPO was of interest in the MA group for further study. The PVN was highly active with many visible c-Fos cells, however these were not readily labelled with NPY.

a) SNO-CFE-MA-PVN



The cell activity in a) the paraventricular of the hypothalamus and b) in the median preoptic of the mouse model *Snord116del* on a chronic treatment of 100mg/kg/d *Caralluma Fimbriata,* after 90 minute administration of beta-mercaptoacetate

b) SNO-CFE-MA-MnPO



The MnPO had less c-Fos activity with only non-labelling fibrous NPY or α- MSH.

Figure 39 Close Up of c-Fos activity and Fibrous Non-labelling
c-Fos (purple):-c-Fos early gene NPY (green): neuropeptide–Y & α-MSH (red): alpha - Melanocyte-stimulating hormone.

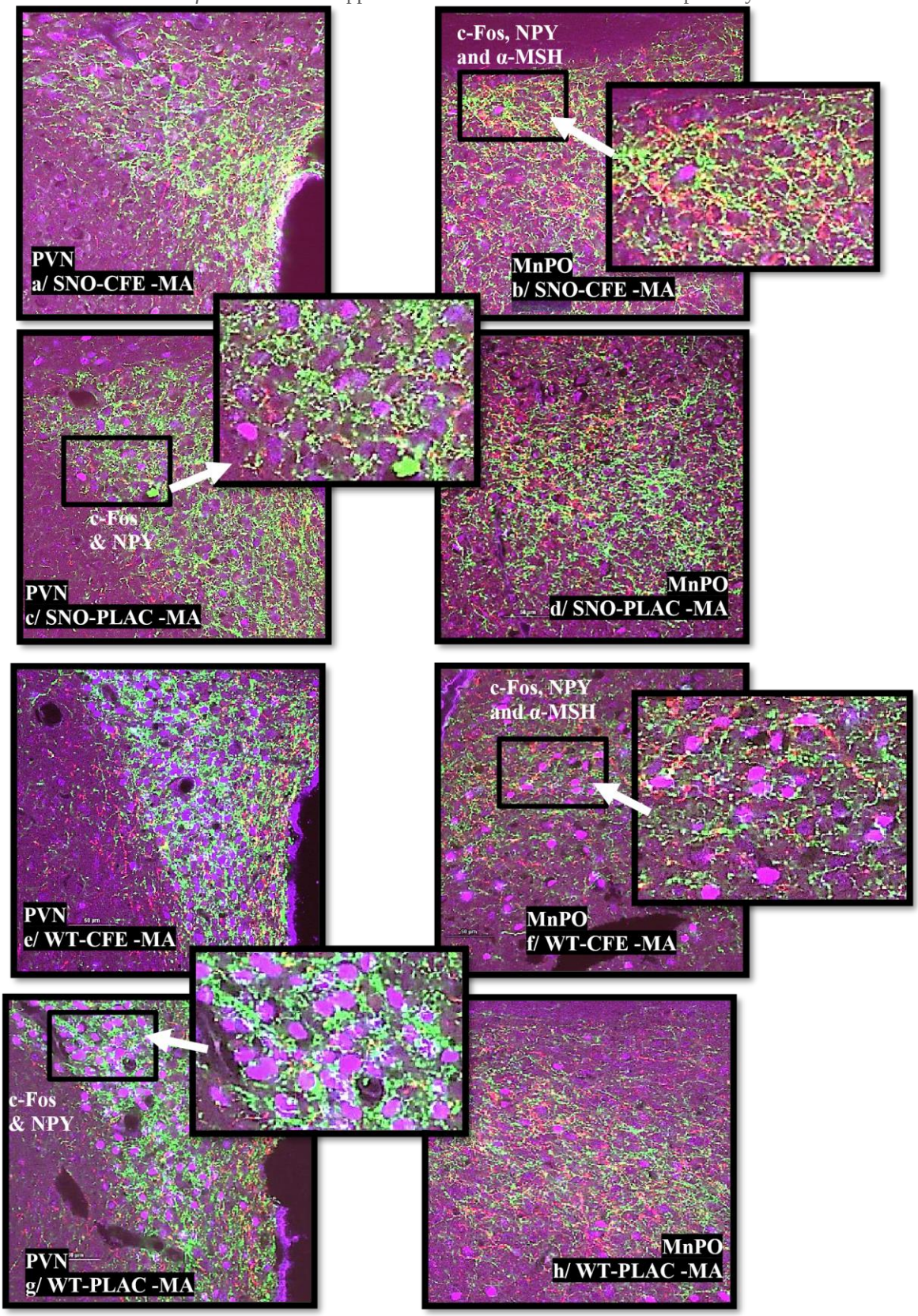


Figure 40 Paraventricular & Median preoptic Co-labelling after Beta-mercaptoacetate Stimulant Immunohistochemistry c-Fos cell activity and fibrous NPY: neuropeptide-Y and α-MSH:- alpha - melanocyte-stimulating hormone, in brain slice scans of the PVN: - paraventricular of the hypothalamus

and the MnPO:- median preoptic of the lamina terminalus in SNO:- *Snord116del* and WT:- wild type mouse model. Animals were ingesting either CFE: - *Caralluma fimbriata* extract, at 100mg/kg/d with the appetite signalling reagents, CFE -PLAC: - maltodextrin/cabbage leaf saline, after 90 minute administration of beta-mercaptoacetate in all groups.

6.5 Discussion

These results of activity in the immunohistochemistry presents a similar picture to the behavioural experiments. Broadly speaking, the cell activity confirmed the behavioural markers of appetite in regards to glucose signalling modification by 2DG (n=5) per group, the SNO mice presented with significantly lower appetite than the WT mice. These significant differences during the 2DG experiments were clearly opposite in action, though in this study they were more obviously seen in the PLAC animal's than the CFE mice. This unusual lower appetite goes against the perception of the *Snord116* deletion animal model experiencing hyperphagia. Yet as was noted in the chronic trials, the hyperphagia in this model was cyclical, so daily intake alters.

Even so, the unusual lowering of appetite due to 2DG, in the SNO animals was not just due to CFE but was more likely a specific strain reaction during glucose deprivation. Reasons for this may be a decreased rate of glucose absorption during what looks like a conservation of energy activity - seen in the behavioural studies - where it was noted that the SNO-CFE animals sat in a still (BALD) posture (Appendix I), during the reduced appetite under glucose deprivation. Another reason for this may be have something to do with the unusual signals seen in the SNO mice.

The results of the MnPO immunohistochemistry for water appetite presented very similar activity to the behavioural studies. These results suggest the SNO-PLAC animals are less thirsty as in the behavioural studies where they drank the least amount of water. It is recently recognized that brain circuits related to body-fluid balance have anatomical connections with areas of the brain concerning reward (Pereira-Derderian et al., 2016). The WT animals had typically increased NPY activity with increased thirst (Pich et al., 1992) which was very similar to the behavioural data. The SNO animals water appetite during stimulation with 2DG was

obviously lower. This presents a connection with glucose signalling and thirst. Naturally fluid intake is connected to food intake. Perhaps these circuits or any connection to reward are damaged in the SNO animals. In the case of the SNO animals (genetic deletion) the drop in thirst was connected to a lower need for glucose demonstrated in the MnPO by both c-Fos activity SNO-PLAC 63.8 ± 17.38; WT-PLAC 84.8 ± 27.34 (P= 0.01), and NPY, SNO-PLAC 12.4 ± 5.22; WT-PLAC 63.4± 37.54 (P= 0.03). The 100CFE - though not significant – it did in this instance enhance the α-MSH signal in the MnPO. In the MA administration similar yet lower values were seen again in the SNO animals.

Therefore, the strains, once again experienced opposite effects i.e. the WT mice had less thirst due to 100CFE. The highest α-MSH co-localization with c-Fos was in the ARC of the WT100CFE-SAL & SNO-100CFE-2DG which though not significant suggests inhibitory activity when including the inhibitory pathway from the ARC to the PVN for α-MSH co-localization of c-Fos activity. Unfortunately, statistical analysis was not relevant in the MA slides due to animal numbers. This study instigates further research in the *Snord116* deletion animals towards an understanding of thirst in humans with PWS.

6.6 Limitations

Unfortunately, for the immunohistochemistry study there was only enough animals and time, to confirm some of the more significant behavioural interactions. The 5-HT2c receptor results warranted a new protocol with more *Snord116del* mice and extra immunohistochemistry to address the need for visual confirmation of the capacity for CFE to interact with the 5-HT2c receptor whilst utilizing the antagonist SB 242084 (Somerville et al., 2007). Therefore, the mechanism of action for CFE is still – some-what - open for interpretation. Though this mechanism is confirmed it may be that, as was said by (Kamalakkannan et al., 2010a) the mechanism is likely to reside along more than one physiological pathway. This inability to study this mechanism by cellular activity was mainly due to the behavioural studies repeatedly using the same animals for each experiment. Therefore, chapter six chose the stimulant which demonstrated the most significant behavioural markers, for cellular appetite mapping. Further the activity related to thirst with results in the MnPO from only one slide per animal, is not a strong indicator to judge the thirst activity. It would have been very interesting to continue the MA studies in this vein, throughout the lamina terminalus.

One further limitation throughout the protocol has been the inaccessibility of accessing blood hormone levels from the animals. When the opportunity for the collection of blood arrived - whilst in the perfusion process – the investigators found the heart rate of the *Snord116del* mice slowed very quickly at a lower level of anaesthetic than the WT. It was expected the investigators would access blood during the perfusion but the animals were too vulnerable at this stage and though the investigators did try more than one process to do this, the perfusions would have suffered for it. Eventually the investigators decided to cease.

6.7 Further study

At this point in time it will clearly be useful to follow through with the immunohistochemistry slides and further identify the role of 2DG and MA in altering the cell activity (to the saline control) within neural networks in the SNO and WT mouse brains. Further research may involve going back to these experiments with another colony of *Snord116del* animals, to clarify the interactions observed during administration of the 5-HT2c antagonist by immunohistochemistry. Though phenotypical disruptions seen in humans with the genetic deletion HBII-85 or

SnoRNA116, may be transferred to the mbii-85/Snord116, this chapter's unusual and opposite cell expression are unusual. This study may inform choices related to the pharmacological regulatory treatment for humans with PWS. Clinical trials have often shown significantly different and alternate outcomes due to the genetics of PWS. Further study needs to address PWS genetics and individualized outcomes due to this genetic deletion, and deprivation by glucose signalling in PWS. Taking this study further it would be important to address the action of other SnoRNA's related to these results. Especially the *Snord115* genetic deletion which is known to actually interact with the *Snord116* area of the critical region seen in figure 1. (Falaleeva et al., 2015). Unfortunately, low viability of the *Snord115* mouse models makes it difficult to investigate serotonin mediation (Kishore and Stamm, 2006, Doe et al., 2009, Morabito et al., 2010). Though ruled out as the primary causal gene for PWS, the importance of the deleted *SnoRNA 115* in humans or in animals as a brain-specific encoder for the 5-HT2c receptor (Cavaillé et al., 2000) is very important. This other deletion may not specifically alter glucose distribution or water appetite but it will be highly likely to contribute to the psychopathologies defined by the 5-HT2c receptor in regards to regulating these parameters

especially in regards to the action of CFE if this is, indeed, in the CNS. Further research may investigate the action of CFE on serotonin-mediated behaviour through the 5-HT2cR addressing the relationship between serotonin, histamine, and sodium intake related to glucose appetite. The first step will be to investigate the hypothesis that, miscoding or ablation of the 5HT2c receptor underlies the maladaptive behaviours associated with PWS. It would be very interesting to do more behavioural type tests to uncover activity related to OCD. Perhaps operant responding tests which allow the animals to establish an association between a particular effortful behaviour (such as lever pressing) to obtain a reward of palatable food. This could investigate serotonin influences on cognition, learning, thermoregulation, sleep, anxiety and appetite (Dimitropoulos et al., 2000, Holland et al., 2003, Benelam, 2009). Leading to immunohistochemistry markers of this stimuli. It is important to recognize that these behaviours moderated by the 5-HT2c receptors are many of the core issues critically demonstrated in PWS (Whittington et al., 2002).

Lastly looking at appetite pathways, there are many other neurotransmitters projected throughout the CNS which are of interest for future study. It would be interesting to know what else is co-localizing with the c-Fos along with NPY or α-MSH. For instance, a central peptide that reduces food directly downstream of the melanocortin receptors is the brain-derived neurotrophic factor (BDNF), as in an earlier figure 11 - though not highly researched within the PWS literature - this peptide's activity is appetite inhibitive and studies investigating a pregnane glycoside-enriched extract - the swamp plant milkweed *Asclepias incarnata* – have been able to increase BDNF downstream of the melanocortin pathway. It is determined that this activity is also able to decrease the secretion of AgRP in the hypothalamus ACN and PVN (Komarnytsky et al., 2013a). Inevitably there will be other peptides of interest within this circuitry. By once again isolating and comparing the activated neurons, engaged in exhibited feeding changes, researchers could further explore regulation of NPY, 5-HT2c (Benelam, 2009, Holland et al., 2003), MC4 receptors and add corticotrophin releasing hormone (CRH), vasopressin mRNA within the PVN and POMC in the anterior pituitary lobe. It may even be possible to establish if CFE interacts through the 5-HT2c receptor, through co-expression or as a ligand, binding within the neuronal pathways.

6.8 Conclusion

The results of the immunohistochemistry activity present a similar picture to the behavioural experiments with strong differences between strains SNO and WT, when ingesting CFE at 100mg/kg/d or PLAC, during stimulation by 2DG in comparison to the control SAL. In answering the core questions of this study, this chapter determines that CFE's reduction in cell activity -related to food appetite - was only minimally noted as in the behavioural studies. The question of how strain alters the neural activity of c-Fos, NPY and α-MSH, in the specific CNS pathways of the ARC and PVN has been answered with stronger activity seen in WT animals compared to the SNO mouse model whose orexigenic immunohistochemistry activity was clearly lower in both parameters of appetite and thirst.

Unexpectedly in this chapter's experiments, the SNO-PLAC, animals had a lower expression of NPY than those ingesting CFE. This was in both groups stimulated by 2DG and the control SAL. Even so, the SNO-100CFE, α-MSH activity in the PVN, determined a stronger inhibitory signal (NS). Over all, this excitation may have balanced the excitatory activity of NPY in the ARC. Even so, overall the brain slice images, none of the α-MSH co-localization signals were significant. On the whole the strongest activity was in the WT-PLAC animals with C-Fos and NPY signalling differences strongly significant in the ARC, between strains, SNO-PLAC-2DG: 49.6 ± 16.68; WT-PLAC-2DG: 218.4 ± 55.38, (P = < 0.001) and the PVN: SNO-PLAC-2DG: 51.0 ± 15.84; WT-PLAC-2DG (n=5) 138.2 ± 49.17, (P = 0.005).

When answering if glucose deprivation by an i.p. injection of the appetite signalling reagent 2DG, altered cellular neural appetite activity in known pathways of the CNS, the immunohistochemistry results confirm the earlier behavioural work, with an opposite than expected trajectory of stimulation between the WT and SNO animals. Once again 2DG lowered the appetite in the SNO animals.

Lastly though there was only one slide per animal, related to thirst - which is not a strong indicator of water appetite - the results in the MnPO demonstrate the lowest parameters of thirst in the SNO-PLAC stimulated by 2DG. This reduced need for water consumption was also indicated in both c-Fos and NPY in the SNO-CFE, though not as strongly. Once again opposite interactions were seen in strains due to CFE with the WT-PLAC animals stimulated by 2DG demonstrating the highest water appetite.

CHAPTER SEVEN

CHAPTER SEVEN

7. 1 Concluding remarks

This thesis defines an opportunity for natural appetite suppression in PWS, through a non-invasive daily supplement. It also determines a mechanism of action for CFE and a pathway for future study of treatments for PWS. The published clinical trial has already penetrated the appetite behaviour in PWS. Since starting this research many individuals with PWS are enjoying an altered lifestyle due to the evidence uncovered in this thesis. Hyperphagia and the associated appetite behaviours are the most chronically disquieting and sometimes alarming contributor to the familial existence. The outcomes of the current research, regarding CFE's regulation through the 5-HT2c receptor, instructs the ability for CFE to be utilized to treat PWS's phenotypical hyperphagia. Further, though characteristics in mice are not fully indicative of how CFE will interact in individuals with PWS, CFE's interchange between energy, appetite and thirst – observed in the mice - is important. More work is needed on the supplement's capacity to focus mood and energy related to food, water and daily activities in PWS.

In regards to the critically important gene *SnoRNA116/HBII-85* and its relationship to NPY and α-MSH neuronal appetite pathways, this study has indeed indicated that the deletion is interacting within this pathway. It also seemed that the appetite behaviour in the animals was more intrinsic than extraneous, i.e. not unduly reward driven. Though both strains were keen to eat the sweet jelly, hunger was able to be reduced in the SNO strain, under different circumstances, especially involving the stimulation of appetite i.e. glucose deprivation. The other interesting aspect was that deprivation created an obsessive behaviour in the mice (spinning upside-down on the cage). This is an interesting observation in regards to this deletion, indications of anxiety or OCD and the complex pathways of appetite. More work may uncover further connections between hunger and obsession or anxiety in PWS.

PWS has many simultaneously non-functioning genes on the paternal chromosome within the critical region a 15q11.2–q13. The *SnoRNA 116* is only one of the deleted genes in a syndrome which affects 1:15,000 – 1:30,000 people worldwide. Importantly those with PWS have complex individualized physical, behavioural and intellectual difficulties, correlating with extremely individualized ages for onset of the phenotypes. Though the severity and onset of the hyperphagia is unpredictable, all with PWS will eventually experience a need for life-long intervention, especially against the prediction of obesity. Also to arrest the behaviours which may be life threatening. It is hoped that further study may determine if there is any relationship between very early intervention – perhaps from newborn to three - with CFE and supressing either the on-set age of hyperphagia or the drive after onset. Perhaps it is possible to alter the trajectory of the long term phenotype. These ideas and others in regards to dose, long term treatment and the capacity for CFE to enhance physical activity may support the road to independence in PWS. Further research, is warranted especially in larger cohorts with PWS.

7.2 A special mention.

The impact of concentrating your scientific research on a personal experience, part of daily life and the continually confronting face of PWS; is not minimal. The balance of work, family and the interactions regarding PWS is fragile and effortful at home. I therefore must once again acknowledge my family Adam, Ruben and Mia. Without them I wouldn't have had the support necessary to work as hard as I have. So heartfelt is my gratitude and love of that little girl at home. Mia's efforts and trust in her family and me are incredible. Mia lives with PWS and fights a pervasive and penetrating range of never ending difficulties, with a smile, with courage and most often without complaint. Mia you are witty, determined and brave and I can't thank you enough for the inspiration and direction you have given my life. I love you, MUM.

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Appendixes:

Appendix A. Inclusion criteria

Table 21 Inclusion Criteria Study One

Recruitment of subject group with Prader-Willi syndrome A & B.	
Participants may experience any of the symptoms of PWS listed below.	
Hypotonia	Reduced or diminished muscle tone which results in problems with strength, coordination and balance.
Developmental delay	Delayed developmental attainment in global areas including physical gross and fine motor, cognitive, social, behavioural or within learning.
Growth Hormone (GH) deficiency or on Growth Hormone Treatment	Body produces less of the hormone which stimulates growth and cell reproduction.
Low sex steroids (testosterone and oestrogen)	Levels of the steroidal hormones may cause a the female menstrual cycle, controlled by the endocrine system, to be delayed or the decreased functional activity of the gonads may have lowered the testosterone levels.
Hypogonadism	Seen in both males and females. Decreased testicle hormone production in males.
Sleep disturbances (not on a respirator)	May include waking during the night, heavy breathing or snoring, sleep walking or short disturbances in sleep which minimise the deep or REM (rapid eye movement) sleep.
Temperature dysregulation	Disturbed thermoregulation may result in a raised temperature during hot weather or cold extremities during winter.
High pain threshold	Able to experience more pain with less discomfort.
Skin picking – cellulitis	This may occur anywhere on the body with the face, arms and legs being the most accessible.
Hypopigmentation	A lightened skin colour.
Dental problems and thick saliva	Capped or extracted teeth and a thicker viscous saliva which may cause crusting around the mouth.
Auditory difficulties or deafness	

Vision impairment	Wearing glasses for difficulties with sight, or having a divergent gaze or squint.
IQs within the range of 40 to 105	
Those with learning issues	These may be difficulties in conceptual understanding, attention and short-term auditory memory.
Autistic tendencies	Any tendencies such as Obsessive Compulsive Disorder (OCD) behaviour, behavioural temper tantrums, re-doing, concerns with symmetry asking and telling(Dykens and Shah, 2003)
Food seeking behaviours	Those with behaviour such as food seeking, manipulation, possessive, stubbornness, hoarding, stealing or lying related to food.
Abnormalities in appetite regulating hormones (Eg. elevated ghrelin)	It was not a requirement for participants within the study to have been tested for any abnormalities in this.
Hyperphagia	A chronic hunger with a delay in satiation.
Slow metabolism	The body utilizes or metabolizes energy (food) less efficiently.
Minimal energy expenditure	Many with PWS may become tired more easily than other people during daily life or during physical activity. This is because of the slowed metabolism and the minimal lean mass (strong muscle).
On medication or natural therapies:	Such as Co-enzymeQ10 or Fish Oil.
PARENT/CARER b/	
English Speaking	Able to understand and fill out the hyperphagia questionnaire
Number One Carer	Constant supervision where possible and in consultation with outside carers I.e. respite workers, teachers, aides and special school facilitators.

Appendix B. Exclusion criteria

Table 22 Exclusion Criteria for Study One

Recruitment for Prader-Willi syndrome subject group A & B	
Any of the criteria or symptoms of PWS listed below:	
Impaired Glucose Tolerance IGT	Those with PWS who have a prevalence of glucose intolerance or are considered to have an impaired glucose metabolism.
Type 1 diabetes	
Type 2 diabetes	.
Insulinemia	
Chronic Asthma	
Allergic reactions to food or supplements	
Metabolic syndrome	Metabolic syndrome is associated with a group of cardiovascular risk factors including, obesity, blood pressure difficulties dyslipidemia, elevated plasma glucose levels. (The National Cholesterol Education Program, 2001).
Past indications of kidney dysfunction	
Liver damage	
Heart disease	
Bariatric surgery.	
Severe sleep-apnoea	The cut-off point to define severity was those on a respirator at night
Those in psychiatric care.	
Those with a recorded history related to their inability to vomit.	
Those with a medically recorded history of reduced pain sensitivity.	(Not anecdotal)
Those people with PWS who are not under supervision.	
Those on medications other than GH and the included natural substances.	These include but are not limited to seizure medication eg: *Phenobarbitone*, attention deficit /hyperactivity disorder (ADHD), eg. *Ritalin*, sex hormones, eg. *Testosterone* and psychiatric disorders medications eg. Tricyclics.

Appendix C. Hyperphagia questionnaire

1) How upset does your child generally become when denied a desired food?

___ Not particularly upset at all

___ A little upset

___ Somewhat upset

___ Very upset

___ Extremely upset

(2) How often does your child try to bargain or manipulate to get more food at meals?

___ A few times a year

___ A few times a month

___ A few times a week

___ Several times a week

___ Several times a day

(3) Once your child has food on their mind, how easy is it for you or others to re-direct your child away from food to other things?

___ Extremely easy, takes minimal effort to do so

___ Very easy, takes just a little effort to do so

___ Somewhat hard, takes some effort to do so

___ Very hard, takes a lot of work to do so

___ Extremely hard, takes sustained and hard work to do so

(4) How often does your child forage through the trash for food?

___ Never

___ A few times a year

___ 1–2 times a month

___ 1–3 times a week

___ 4 to 7 times a week

(5) How often does your child get up at night to food seek?

___ Never

___ A few nights a year

___ 1–2 nights a month

___ 1–3 nights a week

___ 4 to 7 nights a week

(6) How persistent is your child in asking or looking for food after being told "no" or "no more"?

___ Lets go of food ideas quickly and easily

___ Lets go of food ideas pretty quickly and easily

___ Somewhat persistent with food ideas

___ Very persistent with food ideas

___ Extremely persistent with food ideas

(7) Outside of normal meal times, how much time does your child spend talking about food or engaged in food-related behaviours?

___ Less than 15 minutes a day

___ 15 to 30 minutes a day

___ 30 minutes to an hour

___ 1 to 3 hours a day

___ more than 3 hours a day

(8) How often does your child try to steal food (that you are aware of?)

___ A few times a year

___ A few times a month

___ A few times a week

___ Several times a week

___ Several times a day

(9) When others try to stop your child from talking about food or engaging in food-related behaviours, it generally leads to:

___ No distress or upset

___ Mild distress or upset

___ Moderate distress or upset

___ Severe distress or upset

___ Extreme distress, behaviours can't usually be stopped

(10) How clever or fast is your child in obtaining food?

___ Not particularly clever or fast

___ A little clever or fast

___ Somewhat clever or fast

___ Very clever or fast

___ Extremely clever of fast

(11) To what extent to food-related thoughts, talk, or behaviour interfere with your child's normal daily routines, self-care, school, or work?

___ No interference

___ Mild interference; occasional food-related interference in completing school, work, or hygiene tasks

___ Moderate interference; frequent food-related interference in completing school, work, or hygiene tasks

___ Severe interference; almost daily food-related interference in completing school, work, or hygiene tasks

___ Extreme interference, often unable to participate in hygiene tasks or to get to school or work due to food-related difficulties

Additional items:

(12) How old was your child when they first showed an increased interest in food?

(13) How variable is your child's preoccupation or interest in food?

___ Hardly ever varies

___ Usually stays about the same
___ Goes up and down occasionally
___ Goes up and down quite a lot
___ Goes up and down all the time

Appendix D. Investigator Training

Table 23 Investigator training for animal studies

MONASH ANIMAL RESEARCH PLATFORM ANIMAL HANDLING	Restraint of rats and Mice
	Administration of substances
	Anaesthesia for Rodents
TRANSPORT OF DANGEROUS GOODS:	T003# Import, Export, Packaging of Biologicals
	Actions for 'IBC T003# Import, Export, Packaging of Biologicals
OCCUPATIONAL HEALTH AND SAFETY (OHS).	IBC T001# OGTR Compliance
	Actions for 'IBC T001# OGTR Compliance
	IBC T002# Biosafety Compliance
	Actions for 'IBC T002# Biosafety Compliance'
	IBC T004# Biosafety Cabinet Training
	Actions for 'IBC T004# Biosafety Cabinet Training
	IBC T005# Autoclave Training
	Actions for 'IBC T005# Autoclave Training
UNIVERSITY OF MELBOURNE (UOM) FACULTY OF MEDICINE, DENTISTRY AND HEALTH SCIENCES FMDHS OHSE TRAINING:	Chemical management
	Manual handling and ergonomics
	Gas Safety
	Personal Protective Equipment (PPE)

Table 23 presents the training attained during Joanne Grigg's study

Appendix E. Rodent Health Screen Panel

Table 24 Health Screen Panel

Garvan Institute *Snord 116* deletion Mouse Models.	
Health Screen	"MBC panel"
	Mouse model
	Quarantine - $340 per animal tested
Viruses	Mouse Hepatitis Virus Mouse Norovirus Mouse Rotavirus (EDIM) Minute Virus of mice Mouse Parvovirus Pneumonia Virus of mice Sendai Virus Theiler's Murine Encephalomyelitis Virus Ectromelia Virus Lymphocytic Choriomeningitis Virus Mouse Cytomegalovirus Mouse Adenovirus type 1 Mouse Adenovirus type 2 Reovirus type 3 Hantavirus Polyoma Virus K Virus
Parasites	Ectoparasite/endoparasites
Bacteria, mycoplasma and fungi	Bordatella bronchiseptica Citrobacter rodentium Clostridium piliforme (Tyzzer's disease) Corynebacterium kutscheri Mycoplasma pulmonis Pasteurella pneumotropica and other Pasteurellaceae Salmonella spp. Klebsiella pneumonia and K. oxytoca Staphylococcus aureus Pseudomonas aeroginosa Streptococci b-haemolytic (not group D) Streptococcus pneumoniae Helicobacter spp. CAR Bacillus Proteus spp. E.coli E. cuniculi

Table 24 : describes the health screening for the *Snord116* deletion mouse model from the Garvan Institute sent to the Florey Neuroscience Institute animal facility; as sent by the Bioresources Manager.

Appendix F. Generational Mating and Birth Charts

Table 25 First Generation Snord116del and WT mice

Florey Neuroscience Institute 3rd floor facility.

SNO MATING Box	Female	MTD	Male	Litter Born	#	Still Born	W-F	W-M	REMARKS
SNO1	999	20/03/2015	994	10/04/2015	7		5	2	weaned
SNO2	1000		SEP25/5/15	11/04/2015	5		4	1	weaned
				3/05/2015	11		7	4	weaned
				22/05/2015					eaten
SNO3	996	20/03/2015	991	11/04/2015	2				eaten
			SEP25/5/15	1/05/2015	8		3	5	weaned
				25/05/2015	4	1			eaten
SNO4	998	20/03/2015	993						blood in the box 13/4/15
			SEP 1/6/15	1/05/2015	7		5	2	weaned blood in the box 25/5/15
SNO5	997	20/03/2015	992	12/04/2015	9		5	4	weaned 1 female fd 5/5/15
			SEP25/5/15	6/05/2015	9		2	7	weaned
				2/06/2015					eaten
WT M Box	Female	MTD	Male	Litter Born	#	Still Born	W- F	W-M	REMARKS
WT1	73708	16/04/2015	73710	14/05/2015					fd
			SEP 18/6/15	9/06/2015	6		4	2	weaned
WT2	73709	16/04/2015	73711	9/05/2015	7		4	3	weaned
			SEP 18/6/15	1/06/2015					eaten
				23/06/2015	3		1	2	weaned
WT3	73712	16/04/2015	73715	9/05/2015	8		6	2	weaned
			SEP 18/6/15	3/06/2015	8		5	3	weaned
				29/06/2015	3		2	1	weaned
WT4	73713	16/04/2015	73716	10/05/2015	8		4	4	weaned
			SEP 18/6/15	1/06/2015	7		3	4	weaned
WT5	73714	16/04/2015	73717	9/05/2015	5		3	2	weaned
			SEP 18/6/15	18/06/2015	9		5	4	weaned

Tables 25 Animal husbandry tables of mating pairs, births, litter amounts, gender, weaning and animal deaths for both the genetically modified SNO - Snord116 deletion mouse model and the WT – wildtype control. SEP – September; W – weaned; F- female; M – male.

Appendix G. Jelly Volume, Dose, Equipment and Recipe.

G.1 Calculations:

Table 26 Treatment Dose Calculations

Volume of treatment CFE/PLAC per animal utilizing a single gelatine sachet in 125ml of water/H_2O.	
1 $\frac{\text{1 x CFE - 500mg}}{\text{125ml water}}$ x unit of the jelly powder/ per 125ml water	
125ml H_2O vehicle & 500mg CFE = 1ml per 0.25mg	
Dose 1:	100mg/kg = 10mg/100g = 1mg/10g mouse or 2.5mg per 25g mouse.
Making up the Jelly dose 1	100ml H_2O gelatine/saccharine & 500mg of CFE in 25ml H_2O In 500mg is 200 doses of 2.5mg (25gm mouse) 125ml ÷ 200 = 0.625mg dose = per 25g mouse
Dose 2:	33mg/kg = 1/3 of 100mg =3.3/100g =.33/10g mouse or .83 per (25g mouse)
Making up the Jelly for dose 2	Place 500mg of CFE in 75ml H_2O 100ml H_2O gelatine/saccharine & 500mg of CFE in 167 of CFE in 25ml H^2O In 167mg is 200 doses of .83mg (25g mouse) 125ml ÷ 200 = 0.625mg dose = per 25g mouse
Making up the jelly for the placebo	100ml H_2O gelatine/saccharine & 250mg of malto-dextrin/cabbage leaf in 25ml H_2O In 250mg is 200 doses of 1.25mg (25gm mouse) 125ml ÷ 200 = 0.625mg dose = per 25g mouse

Table 26. Defines the dose and the amounts needed to make up the jelly for the trial of *Caralluma fimbriata* extract – CFE; in the Snord116del mouse model; ml - millilitre; mg - milligrams; g – grams; H_2O – water

G.2. Procedure

The procedure of making up the jelly followed several easy steps below:

Equipment: 500mg lockable vegetable capsules, Spatula, Ice tray (2 X 12 wells) with a silicone base to be pressed in from underneath to eject the jelly from the tray.

1. Boil the 100ml of water to 55-60°C. Stir the water vigorously before adding the gelatine powder. If the water was not spinning before adding the powder the gelatine solution would become viscous and clumpy causing an uneven dissolving of the treatment.

2. Dissolve the gelatine (Davis Gelatine, manufactured by GELITA NZ Ltd., distributed by GELITA Australia Pty. Ltd) in 100mls of spinning boiled water. The instructions connoted 1 sachet per 125ml of water and the boiled water was needed to adequately dissolve the gelatine until no granules were visible.

3. Dissolve the treatment and 2% saccharine: Low Calorie Sweetener (250ml liquid sweetener, Sugarless Australia® Pty. Ltd. 4/26 Barry Road, Chipping Norton 2170 NSW Australia www.sugarlessaustralia.com) 0.05mg = 2% in 25ml of cold water. 100ml per 13 wells/ 500mg CFE = 8 drops/10mg Saccharin. Information on saccharine is found in section…

4. Treatment: 1 = 100mg CFE; 2 = 33mg CFE, differed as follows. Both utilized 500mg CFE dissolved in either: 1 = 25ml of cold water or 2 = 75ml of cold water ($1/3^{rd}$ of 100mg treatment in 75mil = 33mg: $1/3^{rd}$ in 25mil). Both mixtures used only 25ml of dissolved CFE poured into the 100ml of gelatine (step 6). In dose 2, 50ml of CFE and water was disposed of.

5. PLAC : the 250mg - malto-dextrin/cabbage leaf capsule was dissolved in the 25ml of water. This was added to the cooled gelatine (step 6).

6. The dissolved gelatine mixture was cooled - so that it would not affect the active treatment and the 25ml of cold water containing one of three treatments (CFE 100mg/33mg or PLAC) was added to 1 in each of the 3 x 100ml mixtures. This made 3 x 125ml gelatine/CFE 33mg/100mg or PLAC

7. This cooled mixture was mixed thoroughly and poured into the ice tray wells - level to the lip of the container creating a convex meniscus (curved upper surface), without flowing onto the container.

8. This filled exactly 13 wells with no fluid left over (9.6ml per 1 well/ 38.5mg of the treatment). As it was possible to define the volume of each well. Once divided, one dose, per 25gm mouse was 0.62gm. As the mice were mostly <25gm, the break down for slicing a single well for the required volume per mouse was as the table below.

9. Lastly the mice were weighed and the treatment volume was defined as per table. per section

Table 27 Volume Per Animal

Treatment jelly for weight range and dose per weight in animals							
Animal weight (g)	<15	17	19	21	23	25	>27
CFE/PLAC (mg)	1.5	1.7	1.9	2.1	2.3	2.5	2.7
Pieces per well.	25.6	21.4	20.2	18	16.7	15.4	14.2
Dose weight (mg)	0.3	0.4	0.45	0.5	0.55	0.6	0.65

Table 27 presents a measure of set jelly/treatment volume per mouse: g - gram; mg – milligram. As a starting point 1 well = 18 slices each slice 2.1mg of treatment approx. 0.5mg of jelly (evenly cut with a blade), which was the dose for 23g mouse. This was altered to suit each animal.

Note: Eventually each time the treatment was made, the study would use only as many wells as was needed per treatment CFE/PLAC cycle and the rest was disposed of.

Appendix H a. Timeline of Animal Experiment

MonSNO	S3/4	WT1	WT2	Tues	S3/4	WT1	WT2	Wed	S3/4	WT1	WT2	Thurs	S3/4	WT1	WT2	Frid	S3/4	WT1	WT2	Sat	S3/4	WT1	WT2	Sund	S3/4	WT1	WT2
Transport				Quarant				Breed																			
Gestation																MATE											
pregnant																wk2											
																wk3				April							
6th				7th				8th				9th				10th				Born Sn1				12th BSn2			
Mother				14th MF				15th MF				16th MF				17th		WT		MF old				19th			
20th MF				21st MF				22nd MF				23rd MF				24th				25thMF				26th			
27th MF				28th MF				29th MF				30th MF				May 1	BSn3			2nd W	MF			W	BSn4		
4th Wean	MF			5th W	MF			6th MAY	MF			7th BC	MF			8th BC	MF			4 BC	MF	WT		10th	MF	MF	
11th BC	MF	MF		12th BC	MF	MF		13th AEC	MF	MF		14th H	MF	MF		15th H	MF	MF		5 Wks	MF	MF		H 1&2	MF	MF	MF
18th H	MF	MF		19th H	MF	MF		20th H	MF	MF		21st H	MF	MF		22nd J	MF	MF		6 Wks	W	MF	MF	jelly	W	MF	MF
25th J	W	MF	WT	26th J	W	MF	MF	27th ST	W	MF	MF	28th CFE	W	MF	MF	29th CFE	W	MF	MF	30th 7wks	W	W	MF	31st	W	W	MF
1st June	BC	W	MF	2nd CFE	BC	W	MF	3rd 1CFE	W	W	MF	4th CFE	H	W	MF	5th CFE	H	W	MF	6th 8wks	H	W	MF	7th	H	W	MF
8th	H	BC	MF	9th 2wk	H	BC	MF	10th 2CFE	H	W	MF	11th CFE	H	H	MF	12th CFE	J	H	MF	13th 9wks	J	H	W	14th	J	H	W
15th	J	H	W	16th	J	H	W	17tCFE	J	H	W	18CFE	ST	H	W	CFE	CFE	J	W	10wks	CFE	J	W	21st	CFE	J	W
22nd	CFE	J	BC	23rd 4wk	CFE	J	BC	24th 4CFE	CFE	BC	BC	25CFE	CFE	ST	W	26th CFE	CFE	CFE	BC	27th	CFE	CFE	BC	28th	CFE	CFE	H
29th	CFE	CFE	H	30 5wk	CFE	CFE	H	JULY 1st	CFE	CFE	H	2nd	CFE	CFE	H	3rd	CFE	CFE	H	12 wks	CFE	CFE	H	5th	CFE	CFE	J
6th	CFE	CFE	J	7th Fc	CFE	CFE	J	8th 6wFc	CFE	CFE	J	9th Fc	CFE	CFE	ST	10th Fc	CFE	CFE	CFE	11th	CFE	CFE	CFE	12th	CFE	CFE	CFE
13th CFE	CFE	CFE	CFE	CFE	CFE	CFE	CFE	15th2DG	CFE	CFE	CFE	16th	CFE	CFE	CFE	CFE	CFE	CFE	CFE	14wks	CFE	CFE	CFE	19th	CFE	CFE	CFE
20th CFE	CFE	CFE	CFE	21st	CFE	CFE	CFE	CFE MA	CFE	CFE	CFE	CFE	CFE	CFE	CFE	24th	CFE	CFE	CFE	BL	CFE	CFE	CFE	BL	CFE	CFE	CFE
27t 5HT	CFE	CFE	CFE	28th	CFE	CFE	CFE	29th	Fc	CFE	CFE	50%	Fc	CFE	CFE	50%	Fc	CFE	CFE	150%	CFE	CFE	CFE	50%	CFE	CFE	CFE
2nd 50	CFE	CFE	CFE	3rd 50%	CFE	CFE	CFE	CFE	2DG	Fc	CFE	6th	CFE	Fc	CFE	7th	CFE	Fc	CFE	8th	CFE	CFE	CFE	9th	CFE	CFE	CFE
10th CFE	CFE	CFE	CFE	11tACC	CFE	CFE	CFE	12thACC	MA	2DG	CFE	PU	CFE	CFE	CFE	14tPU	CFE	CFE	CFE	15th	BL	CFE	CFE	16th	BL	CFE	CFE
17th	5HT	CFE	CFE	18th	CFE	CFE	CFE	19th	CFE	MA	Fc	20th	50%	CFE	Fc	21st	50%	CFE	Fc	22nd	50%	BL	CFE	23rd	50%	BL	CFE
24th	50%	5HT	CFE	25th	50%	CFE	CFE	26th	CFE	CFE	2DG	27th	CFE	50%	CFE	28th	CFE	50%	CFE	29th	CFE	50%	CFE	30th	CFE	50%	CFE
31st	ACC	50%	CFE	1st SEP	ACC	50%	CFE	2nd	ACC	CFE	MA	3rd	PU	CFE	CFE	4th	PU	CFE	CFE	5th		CFE	BL	6th		CFE	BL
7th		ACC	5HT	8th		ACC	CFE	9th		ACC	CFE	10th		PU	50%	11th		PU	50%	12th			50%	13th			50%
14th			50%	15th			50%	16th			CFE	17th			CFE	18th			CFE	19th			CFE	20th			CFE
21st			ACC	22nd			ACC	23rd			ACC	24th			PU	25th			PU	26th				27th			

Table 28 Animal Group Time table

Appendix H b. Time table Colour Code

Label and Code

SNOS		**Gestation**	**G**	**Wean**	**W**	**Jelly**	**J**	**Feacal**	**Fc**	**5-HT2c Antag**	**5-HT**	**Acclimitaize**	**ACC**
Wild T.	**WT**	**Born**	**B**	Basic chow	BC	**Start CFE**	**ST**	**2-Dioxygl**	**2DG**	**Baseline**	**BL**	**Perfusion**	**PU**
Mate M		**Mother fed**	**MF**	**Handle**	**H**	**CFE**	**CFE**	**Beta-Mercap**	**2MA**	Fast 50%	**50 %**	Jo early	

Table 28 Above is the code for the Animal Group Time table. Each colour relates to the activity of the investigator associated with the protocol for each group.

Appendix I. Animal Characteristics

a/ Phenotypical and behavioural characteristic

At four – six weeks the *Snord116del* mice were extremely active. This jumpy activity seemed even stronger in the SNO mouse model compared to the C57BL/6 WT strain which meant the SNO mice' muscle tone characteristically compared to many other PWS mouse models. "Failure to thrive" was not seen in the early weeks of our study.

During the early procedures of husbandry what was observed, however – noted by the FNI animal house handler – was that the SNO mothers (one in particular) were culling their litters by eating them. In one case the mother ate all the newborn pups. It was also remarked upon that each litter of the *Snord116del* mice had one runt in each litter box. These animals were very tiny at birth and were given extra seed to survive once weaned. Due to their size these animals were not utilized in the experimental protocol but interestingly over the full time period when maintained but not handled they grew at the same rate as the other animals to become very small adult mice.

Before being single housed all the animals were very capable of jumping out of the cage when the wire mesh was lifted and most of the SNO animals were uncomfortable when first single housed. This is presumed due to the fact that the first measures of FLO demonstrated minimal ingestion of food in the first days of single caging for the SNO animals which was absent in the WT. It was also noted that some animals lost weight from when they were randomly assigned

to their groups which affected the balance of group weights only minimally at baseline. Most of these animals gained this weight again shortly afterwards.

During all weighing and handling processes in the experimental protocol - after the first few days - the SNO animals were more cooperative than and not as aggressive as the WT mice. The early chirpy daily activity seemed to slow down by the age of seven weeks. This was in all animals so this was not due to the treatment or animal model. Stress was reduced in the animals handled by the investigator to the point that many of the mice (especially the SNO model) were capable of sitting on the investigators hand without restraint. Even so there were some animals who always needed restraint SNO-IF-100CFE was particularly fast and SNO-5M-33CFE was

especially calm. The latter mouse did not need restraint even after injections. Therefore, it was clearly demonstrated by daily interactions, that the mice had both individualized characteristics and characteristics relative to the mouse model or treatment. On the whole, though SNO animals were gentler in nature than the WT, CFE altered the speed by which the animals moved. Those ingesting CFE jelly were more hyperactivity relative to dose. This was without creating more aggression. The only time the SNO animals showed aggression similar to practically all the WT mice, was in one case during the perfusion protocol when frightened.

b/ Jelly dosing

In regards to the method of administering the treatment all the mice were similar in characteristics. By the second day of training >95% of the mice commenced to eat the jelly within 1 minute after the jelly presentation and often completed the jelly in one sitting. There was momentary movement exploring the cage but they would rarely leave the treatment without finishing. The investigator did find minimal and random corners of hardened jelly in their houses but on close to all occasions >95% the jelly was eaten. Therefore, throughout the entire study it seemed obvious that all the animals enjoyed sweetened food.

On the whole the administration of CFE made the animals less likely to enjoy being handled, harder to catch in their houses and obviously more hyperactive. During the 4hr appetite protocol (reading within the start of the dark cycle) at least 50% of the 100CFE SNO and the 33CFE WT were fast enough to be a blurred movement in the cage. This was however altered in the SNO animals during the 2DG experimental protocol.

These animals were noted but there was never one animal in particular the occasions were rare. On the fourth day to help train the animals for treatment feeding the PLAC was added into the 2% saccharin at a volume determined by the weight of each animal. In all cases this more bitter tasting jelly was also eaten.

The PLAC animals were mainly asleep during the light cycle and fast moving during the dark cycle. The CFE animals were hyperactive when picked up and extremely fast during the dark cycle (especially the SNO, CFE100mg). Though faster these animals were not aggressive. However, the WT animals from all groups were more likely at all times to bite if aggravated.

c/ Animals levels of hunger during daily treatment.

On the whole the investigator maintained an average of 18gms in the lid for consumption. In regards to the consumption there was a difference over all in the animals' appetite homeostasis. The SNO animals cycled from high, medium to low in the amount they ingested. Not all in the exact same cycle but clearly all animals' appetite differed from day to day in cycles with unexpected days of hyperphagia. In comparison the WT animals' daily amount consumed, stayed fixed at a similar amount. This amount was eaten from day to day with no obvious change. Their appetite was more balanced on the whole. Unusually those animals on 33CFE/kg/d ate less than those on the 100CFE/kg/d. Please note the discussion in regards to this unusual result.

When including the lower weight of the SNO mice, the results of the chronic feeding study determined that they were hyperphagic. Their lower weight meant they should've eaten less but when adding the -5% weight adjustment in the SNO mice, the SNO animals ate significantly more in the CFE groups and PLAC group. The SNO mice also reduced their amount consumed over the eight-week period in a ratio relative to their dose/or not of CFE. Therefore, though not significant the study did determine a dose response and trend in attenuation of the hyperphagia due to CFE, in the SNO animals as compared to the WT.

SNO animals clearly cycled through appetite levels no matter what treatment. The cycles - though still there - were lower in the 100CFE/kg/d compared to the 33CFEkg/d and further compared to the PLAC group on PLAC which was the highest. At first some of the SNO animals seemed to go through cycles of hyperphagia. This meant that every now and then they would consume at least a 3rd more against their average daily consumption during one dark cycle; eating up to 8g/d in one instance. On the occasion that a SNO animal had such a high hyperphagic day (stronger appetite), this seemed to be followed by a low amount eaten on the next day. The wild type animals had their highest daily amount measured (not during 50% food deprivation test) as 4.6g/d. This was from one particular animal who was on the whole a higher consumer of food. On the whole for the SNO animals this hyperphagic drive did not continue throughout the treatment protocol. Regarding consuming the treatment both the SNO & WT mice would go to feed on the jelly straight after it was inserted into the cage.

d/ Animals during saline control.

As expected there were no unusual alterations in the reactions of the animals during the control injection procedures. After i.p injections all the animals returned to their houses and ate or drank randomly until once again settling. This led to sleeping until the dark cycle propelled their appetite as on any other routine day. The SNO animals did eat more from being woken at this stage.

e/ Animals during glucose deprivation

Observations determined a very strong and clear difference in the reaction to 2DG as a behavioural response to the i.p. injections. This response however was not as noticeable in the second litter groups so therefore the statistical analysis, does not fully distinguish the initial differences seen in the groups. This may have been due to parentage.

These behavioural characteristics had three levels in the SNO animals and a different three levels in the WT. These levels were

1) "Balled" (BLD): the animals were inactive and still. There was a behavioural somnolence as they were curled up in a ball, with no activity towards food. This stillness was not a sleeping position i.e. not in their tissued beds. They were instead upright in a round position with their heads low, placing themselves in obscure angles within the cage, i.e. no protectively or close to the edge of the box and not near the food. They were mainly in the middle of their houses unprotected. When interrupted the animals would start and take a couple of steps (only) until still once again in a balled position.

2) "Balled but active" (BDA) as above but not as distinctive with more movement around the cage – not just when startled. At times to eating and/or drinking

3) "active and eating (ACE).

After the i.p. injection of 2DG; in the first group the SNO-100/kg/d CFE were BLD by 30 - 45 mins. This behaviour did not change much throughout the four-hours during observations, though the animals did move to drink. At moments they went to eat but this was minimally noted. This BLD behaviour was not so clearly experienced in second cohort. It is not clear why. The CFE33/kg/d SNO animals were BDA, similarly balled yet the reaction was not as strong

as the 100CFE/kg/d mice. They voluntarily, yet sporadically ate and they drank a lot of water over the 4 hr timeline. They were however less hungry than was expected after being administered the 2DG appetite stimulant. Though active, after eating they would settle near or in their tissue beds. There was however one mouse who fully acted BLD like the 100CFE group during the experimental timeline.

Lastly the SNO/PLAC, animals started feeding within 45 minutes of being injected with 2DG. There seemed to be a constant interest in food but this interest was not as strong as in the WT animals. On the whole the SNO/PLAC were ACE, actively eating and drinking throughout the time period.

Unfortunately, this clear cut division was not so remarkable in the second group of SNO animals so the within group statistics are not as significant as expected by the first observations. The reason for this is not clear. The other observation is that this BLD way of being may have been a SNO characteristic.

In comparison the WT animals were all ACE. Observationally there were levels of hunger but not with such clear cut lines. There was still a suspected suppression of appetite due to CFE but this was not observable as all the animals seemed affected by the 2DG. In fact the CFE33kg/d group seemed hungrier to the eye. This looked like a continual ACE mode, to the point that they stayed next to the food and continually ate. However, the appetite calmed during the dark cycle in the last hour. Over all statistically the WT/PLAC group ate constantly.

f/ Animals during Fatty acid signalling tests

The appetite behaviour observed during the MA i.p. injections protocol was very different than during the 2DG protocol. At one hour after the injections all the SNO animals were sleeping and eating on occasion. This activity was observed throughout the four-hour experiment, but most of the food eaten was during the dark cycle. The second group of PLAC animals were not quite as interested as the first as they seemed to sleep more with sporadic feeding. Interestingly the WT animals were similar but the WT 33/kg/d CFE group were not hungry at all. They continued to sleep in their tissued beds. This did not look the same as the BLD posture.

g/Animals during 5-HT2c antagonist experiment

During the four-hour observation period after the administration of SB 242084 - as opposed to the 2DG and MA experiments - the SNO groups all seemed similar in their reaction. All the SNO CFE and PLAC animals were actively hungry and seeking food with sporadic rests in between. This continued into the dark cycle but not furiously. They did not show any signs or symptoms of unusual jumpy behaviour, more just a continual need for access of their food. The PLAC group ate by observation. After the experimental protocol on the whole, the mice were very quiet the next day and they also didn't eat very much.

Similarly, the WT SB 242084 groups were eating after having had the i.p. injection, except the largest 100CFE animal. This animal seemed to stay still. However, other than this animal though all the animals seemed hungry most of the WT animals were asleep by 1hr after administration. This was then followed by sporadic eating, sleeping and stillness with what looked like a shaking through their bodies in many of the animals. The WT 100CFE mice seemed somehow more-jumpy than the others and unusually the WT 33CFE were burying in the ground. At the end of the four hours in the dark cycle the WT 100CFE and 33CFE groups were unusually quiet during the weighing. The PLAC group seemed less affected and interested in food overall. The next day the WT33CFE groups were walking, what could be considered as timidly? The PLAC animals were more energetic than the others but on the whole the WT animals did not seem hungry.

h/ Animals during 50% fast

On average most of the animals showed distress after the 3rd day of 50% food deprivation. It is know that if there is a scarcity of food and a loss of weight the temperature of the body will fall if there is not enough fat within the animal to consistently keep the glucose to the brain. The increased metabolic action will take effect in the mice causing a pilo erection of the hair on the animal's bodies (like a shivering response in humans). This was seen in most of the animals with the "Balled" pose prominent in all groups. This pose was a curled up position with piloerection of the fur.

The differences were mainly in the reactions between this balled effect depending on the treatment and the strain. As mentioned by day four the protocol was stopped. The ethically

approved fifth day of 50% deprivation was called off and all the animals were fed their premeasured food. Fluid was fully available throughout the fast.

For all animals it was clear that by day 2 there was less activity. Some of the SNO 100CFE, 33CFE and PLAC showed signs of distress already. This distress was observed in all the SNO animals by day 3. Though the WT animals were very still and quiet it was not until day four that they showed behaviours which the investigators perceived as distress. However, though these animals were similarly distressed - as the SNO - they did not exhibit the same behaviour. The signs of distress in the SNO animals were as follows: all the animals would climb to the cage wire on the top of their houses and would spin in circles. This looked like a shelf of small upside down spinning fans (nonstop). This behaviour could have been considered very OCD as the animals would then get down again and stay very still in a small ball for approximately 10 seconds and then the mice would once again climb to the wire mesh and spin very fast upsidedown. Different to this a few of the WT animals would climb onto their wire and climb down again. There was no spinning behaviour.

On day four the investigators believed the protocol needed to be stopped. One 100CFE animal seemed very ill and BALD with a swollen face. The vet was called in and the protocol was stopped. It was believed that feeding would arrest this development. The ill animal was given some weighed food and within a short period the mouse was able to move around to continue to eat. It seemed clear that the SNO 100CFE animals were more likely to become ill.

On the whole the WT CFE were less likely to jump into the food straight away at the end of the protocol. They were very quiet but not so BALD. One animal, however, of the WT 100CFE group was retired from the protocol at day three, due to having lost a greater amount of weight. On the whole, all animals lost weight quickly but none lost 10% of the weight measured the day before. This weight was gained within the two days of completion. On the fourth night of the 50% protocol when the pre-measured ad libitum food was offered the amount eaten was started immediately. At first carefully in those animals who were becoming ill but in all animals the premeasured food eaten continued into the dark cycle.

One last note on behaviour; interestingly when the animals were left alone after all the protocols - over the last week before the perfusions - they all seemed to lose weight. There is no clear

reason for this except perhaps they were woken less during the light cycle and therefore left their chow till the dark cycle.

Appendix J a. Mean and Standard deviation study two

Table 29 Mean and Standard Deviation Appetite Signalling

Two-Way MANOVA comparisons of food eaten over appetite signalling tests, between strain: Snord116del and C57BL/6 wild type control mouse model when treated with Caralluma fimbriata extract or a control placebo.

Stim.	Strain	Treat	Mean	SD	N	Reagent	Strain	Treat	Mean	SD	N
		100CFE	1.21g	0.27g	12			100CFE	0.77g	0.37g	12
		33CFE	1.47g	0.23g	12			33CFE	0.98g	0.28g	12
	SNO	BC	1.46g	0.41g	12		SNO	BC	1.00g	0.25g	12
		Total	1.39g	0.32g	36			Total	0.92g	0.31g	36
		100CFE	0.82g	0.27g	12			100CFE	1.19g	0.18g	12
		33CFE	0.94g	0.30g	12			33CFE	1.35g	0.30g	12
Saline	WT	BC	0.91g	0.39g	12	2DG	WT	BC	1.55g	0.67g	12
		Total	0.89g	0.32g	36			Total	1.36g	0.45g	36
		100CFE	1.03g	0.34g	24			100CFE	098g	0.35g	24
		33CFE	1.20g	0.38g	24			33CFE	1.17g	0.34g	24
	Total	BC	1.18g	0.48g	24		Total	BC	1.28g	0.57g	24
		Total	1.14g	0.40g	72			Total	1.14g	0.44g	72
		100CFE	0.91g	0.34g	12			100CFE	1.46g	0.38g	12
		33CFE	1.05g	0.36g	12			33CFE	1.60g	0.64g	12
	SNO	BC	0.96g	0.31g	12		SNO	BC	1.55g	0.47g	12
		Total	0.97g	0.34g	36			Total	1.53g	0.50g	36
		100CFE	1.06g	0.16g	12			100CFE	1.61g	0.61g	12
		33CFE	0.86g	0.33g	12	SB		33CFE	1.78g	0.70g	12
MA	WT	BC	1.03g	0.45g	12	242804	WT	BC	1.25g	0.44g	12
		Total	0.98g	0.34g	36			Total	1.54g	0.62g	36
		100CFE	0.99g	0.27g	24			100CFE	1.53g	0.50g	24
		33CFE	0.95g	0.35g	24			33CFE	1.69g	0.66g	24
	Total	BC	1.00g	0.38g	24		Total	BC	1.40g	0.47g	24
		Total	0.98g	0.33g	72			Total	1.54g	0.56g	72

Table 29 Mean and SD - standard deviation comparisons of food eaten in grams, after 4 hrs, due to signalling reagents SAL: - saline control, 2DG: - 2-deoxy-glucose, MA: - beta – mercaptoacetate or the 5-HT2c antagonist SB242804 on the Garvan *Snord116del* mouse model (SNO) and C57BL/6 wild type (WT) strains on either of two doses of treatment CFE: - *Caralluma fimbriata* extract or PLAC: – placebo of maltodextrin/cabbage leaf.

Appendix J b. Appetite and thirst stimulant and deprivation t-tests

Table 30 Mean and Standard Deviation Water Appetite

Two-Way MANOVA comparisons of fluid intake during appetite signalling tests between strain: *Snord116del* and C57BL/6 wild type control mouse model when treated with Caralluma fimbriat extract or a control placebo.

Stim.	Strain	Treat	Mean	SD	N	Reagent	Strain	Treat	Mean	SD	N
Saline	SNO	100CFE	6.24ml	2.71ml	12	2DG	SNO	100CFE	6.54ml	3.02ml	12
		33CFE BC	7.24ml	2.28ml	12			33CFE BC	6.16ml	3.91ml	12
		Total	5.85ml	1.40ml	12			Total	4.85ml	4.97ml	12
	WT	100CFE	6.44ml	2.22ml	36		WT	100CFE	5.85ml	4.00ml	36
		33CFE	3.81ml	1.48ml	12			33CFE	4.16ml	1.35ml	12
		BC	4.15ml	1.81ml	12			BC	4.47ml	1.46ml	12
			5.79ml	1.49ml	12				5.10ml	2.63ml	12
		Total	4.58ml	1.78ml	36			Total	4.58ml	1.89ml	36
		100CFE	5.03ml	2.47ml	24			100CFE	5.35ml	2.59ml	24
		33CFE	5.70ml	2.56ml	24			33CFE	5.31ml	3.01ml	24
		BC	5.82ml	1.41ml	24			BC	4.98ml	3.89ml	24
	Total	Total	5.51ml	2.21ml	72		Total	Total	5.21ml	3.17ml	72
MA	SNO	100CFE	2.62ml	1.64ml	12	SB 242804	SNO	100CFE	5.84ml	1.74ml	12
		33CFE	1.80ml	1.72ml	12			33CFE	6.29ml	2.78ml	12
		BC	2.89ml	2.55ml	12			BC	3.84ml	0.92ml	12
		Total	2.44ml	2.01ml	36			Total	5.32ml	2.19ml	36
	WT	100CFE	5.16ml	2.47ml	12		WT	100CFE	6.69ml	1.48ml	12
		33CFE	6.12ml	1.61ml	12			33CFE	8.36ml	3.42ml	12
		BC	5.16ml	1.27ml	12			BC	4.76ml	1.66ml	12
		Total	5.48ml	1.86ml	36			Total	6.60ml	2.73ml	36
	Total	100CFE	3.89ml	2.43ml	24		Total	100CFE	6.26ml	1.63ml	24
		33CFE	3.96ml	2.74ml	24			33CFE	7.32ml	3.23ml	24
		BC	4.02ml	2.29ml	24			BC	4.30ml	1.40ml	24
		Total	3.96ml	2.46ml	72			Total	5.96ml	2.54ml	72

Table 30 Mean and SD - standard deviation comparisons of fluid (water) ingested in millilitres, after 4 hrs. Scores are due to the appetite signalling reagents SAL: - saline control, 2DG: - 2-deoxy-glucose, MA: - beta – mercaptoacetate or the 5-HT2c antagonist SB242804 on the Garvan *Snord116del* mouse model (SNO) and C57BL/6 wild type (WT) strains on either of two doses of the treatment CFE: - *Caralluma fimbriata* extract or PLAC: – placebo of maltodextrin/cabbage leaf.

Table 31 Saline Food and Fluid

Control unpaired *t* – test, food and fluid ingested over 4hrs during i.p. injections of saline in the *Snord116del* against C57BL/6 wild type mouse model on treatment *Caralluma* fimbriata or placebo.

SAL	SNO (n=12)			WT (n=12)		Unpaired t-test
FOOD	Mean	SD	P value	Mean	SD	*P* value
100CFE	1.21	0.27	0.81		0.27	0.001**
33CFE	1.47	0.23	0.94		0.3	<0.001**
PLAC	1.46	0.41	0.91		0.39	0.003**
t-test	SNO-SNO			WT-WT		
100-33			0.03*			0.32
100-PLAC			0.13			0.51
33-PLAC			0.94			0.84
FLUID	SNO (n=12)			WT (n=12)		t-test
100CFE	6.24	2.71	3.81		1.48	0.01*
33CFE	7.24	2.28	4.15		1.81	0.003**
PLAC	5.85	1.49	5.78		1.48	0.92
t-test	SNO-SNO			WT-WT		
100-33			0.34			0.61
100-PLAC			0.66			0.004**
33-PLAC			0.08			0.02*

Table 31 Saline unpaired *t* –tests, mean and standard deviation (SD) of food and fluid ingested over 4 hours from baseline, in the SNO: Garvan *Snord116del* mouse model against C57BL/6, WT: wild type strain. Mouse models received intraperitoneal injections of saline in groups. These groups were categorized by within strain factors of treatment CFE: extract of *Caralluma* fimbriata x 2 doses (100mg/kg/d & 33mg/kg) against PLAC: placebo of maltodextrin/cabbage leaf. Saline: SNO compared to WT plus within strain treatment group results.

Table 32 2-Deoxy-Glucose Four-Hour Food and Fluid Study

Unpaired *t* – test, food and fluid ingested over 4hrs after i.p. injections of 2-Deoxy – Glucose or saline in *Snord116del* against C57BL/6 wild type mouse model on treatment *Caralluma* fimbriata or placebo.

2DG	SNO (n=12)			WT (n=12)		t-test
FOOD	Mean	SD	*P* value	Mean	SD	*P* value
100CFE	0.78	0.37		1.19	0.18	0.002**
33CFE	0.98	0.29		1.36	0.3	0.005**
PLAC	1.01	0.26		1.56	0.67	0.01*
t-test pairing	SNO-SNO			WT-WT		
100-33			0.14			0.13
100-PLAC			0.09			0.08
33-PLAC			0.84			0.35
FLUID	SNO (n=12)			WT (n=12)		t-test
100CFE	6.54	3.0		4.17	1.35	0.02*
33CFE	6.16	3.9		4.47	1.46	0.17
PLAC	4.85	5.0		5.11	2.63	0.87
t-test pairing	SNO-SNO			WT-WT		
100-33			0.79			0.60
100-PLAC			0.32			0.38
33-PLAC			0.48			0.29

Table 32 Unpaired *t* –tests, mean and standard deviation (SD) of food and fluid ingested over 4 hours from baseline, in the SNO: Garvan *Snord116del* mouse model against C57BL/6, WT: wild type strain. Mouse models received intraperitoneal injections of 2DG: 2-Deoxy-D-glucose – glucose deprivation - in groups. These groups were categorized by within strain factors of treatment CFE: extract of *Caralluma* fimbriata x 2 doses (100mg/kg/d & 33mg/kg) against PLAC: placebo of maltodextrin/cabbage leaf or 2DG: SNO compared to WT in treatment groups.

Table 33 Beta-Mercaptoacetate Four Hour Food and Fluid study

Unpaired *t* – test, food and fluid ingested over 4hrs after i.p. injections of beta–mercaptoacetate MA, or saline in *Snord116del* against C57BL/6 wild type mouse model on treatment *Caralluma* fimbriata or placebo.

MA	SNO (n=12)			WT (n=12)		*t*-test
FOOD	Mean	SD	*P* value	Mean	SD	*P* value
100CFE	0.92	0.34		1.07	0.16	0.18
33CFE	1.05	0.37		0.86	0.33	0.19
PLAC	0.94	0.31		1.04	0.45	0.63
t-test pairing	SNO-SNO			WT-WT		
100-33			0.35			0.07
100-PLAC			0.74			0.84
33-PLAC			0.52			0.32
FLUID	SNO (n=12)			WT (n=12)		*t*-test
100CFE	2.62	1.64		5.15	2.47	0.009**
33CFE	1.8	1.72		5.87	1.61	<0.001**
PLAC	2.88	2.56		5.16	1.27	0.01*
t-test pairing	SNO-SNO			WT-WT		
100-33			0.25			0.27
100-PLAC			0.76			0.99
33-PLAC			0.23			0.12

Table 33 Unpaired *t* –tests, mean and standard deviation (SD) of food and fluid ingested over 4 hours from baseline, in the SNO: Garvan *Snord116del* mouse model against C57BL/6, WT: wild type strain. Mouse models received intraperitoneal injections of MA beta – mercaptoacetate, (fatty acid signalling) in groups. These groups were categorized by within strain factors of treatment CFE: extract of *Caralluma* fimbriata x 2 doses (100mg/kg/d & 33mg/kg) against PLAC: placebo of maltodextrin/cabbage leaf and comparing strain MA: SNO compared to WT within treatment groups

Table 34 5-HT2C Antagonist Four-Hour Food and Fluid study

4HRS SB 242804	SNO (n=12)			WT (n=12)		*t*-test
FOOD	Mean	SD	*P* value	Mean	SD	*P* value
100CFE	1.46	0.38		1.61	0.61	0.48
33CFE	1.6	0.64		1.79	0.7	0.52
PLAC	1.55	0.47		1.25	0.44	0.12
t-test pairing	SNO-SNO			WT-WT		
100-33			0.52			0.51
100-PLAC			0.62			0.11
33-PLAC			0.81			0.03*
FLUID	SNO (n=12)			WT (n=12)		*t*-test
100CFE	5.84	1.74		6.69	1.47	0.21
33CFE	6.29	2.77		8.37	3.42	0.17
PLAC	3.84	0.92		4.76	1.66	0.13
t-test pairing	SNO-SNO			WT-WT		
100-33			0.64			0.16
100-PLAC			0.002**			<0.001**
33-PLAC			0.008**			0.01*

Table 34 Antagonist unpaired t –tests, mean and standard deviation (SD) of food and fluid ingested over 4 hours from baseline, in the SNO: Garvan Snord116del mouse model against C57BL/6, WT: wild type strain. Mouse models received intraperitoneal injections of 5-HT2c receptor antagonist SB 242804 in groups. These groups were categorized by within strain factors of treatment CFE: extract of Caralluma fimbriata x 2 doses (100mg/kg/d & 33mg/kg) against PLAC: placebo of maltodextrin/cabbage leaf and in strain comparisons during i.p. SB 242804: SNO compared to WT strain treatment group results.

Table 35 Four day 50% Deprivation study

50%	**SNO**	(n=12)		**WT**	(n=12)	*t*.test
FOOD	Mean	SD	*P* value	Mean	SD	*P* value
100CFE	6.69	0.75		7.21	1.52	0.3
33CFE	7.28	0.84		6.72	0.84	0.12
BC	6.82	0.92		7.18	1.05	0.38
	SNO-SNO			**WT-WT**		
100-33			0.88			0.33
100-BC			0.73			0.95
33-BC			0.22			0.25
DRINK	**SNO**			**WT**		*t*.test
100CFE	9.07	1.48		11.82	2.92	0.008**
33CFE	8.22	2.81		12.7	1.63	<0.001**
BC	8.09	2.08		11.52	2.08	<0.001**
	SNO-SNO			**WT-WT**		
100-33			0.36	0.36		0.39
100-BC			0.26	0.24		0.77
33-BC			0.75	0.75		0.15

Table 35 50 % deprivation unpaired *t* –tests, mean and standard deviation (SD) of food and fluid ingested after four days, in the SNO: Garvan *Snord116del* mouse model against C57BL/6, WT: wild type strain. These groups were categorized by within strain factors of treatment CFE: extract of *Caralluma* fimbriata x 2 doses (100mg/kg/d & 33mg/kg) against PLAC: placebo of maltodextrin/cabbage leaf

Appendix K. Perfusions

K a. Intraperitoneal Injection Procedure

Intraperitoneal administration followed a standard operating procedure (SOP) for mice (# TECH 10, University of Columbia Animal Care Centre), for all injections. As many of the animals were small a 27g needle was chosen with an appropriate length of 1ml. Once the dose was designated for each animal the syringes were prepared with the designated amount of reagent placed in a recommended volume of sterile saline. This was drawn up into the syringe and was checked by the other investigator. These were allowed to sit at room temperature to warm as cold injections may startle the animals. The fluid was checked for any air bubbles, which when found were tapped to the centre of the needle and released from the syringe. The mouse was then scruffed by the non-dominant hand, allowing the animal to pull out on the cage first so as the neck was lengthened before scruffing. The mouse was tilted backwards toward the ground which allowed the abdominal viscera to shift towards the head of the animal. This was a helpful procedure to stop any puncturing of abdominal organs when injecting into the ventral side of the animal. The lower right abdominal quadrant was then targeted for injection. The syringe was angled towards the upper body at a 30-40^{o} angle and on penetrating the abdomen the administering investigator slightly pulled back on the syringe to check that the needle had not penetrated any internal organs i.e. the intestines. The animal was then placed back in their housing for the continuation of the experimental protocol whilst being observed for complications.

K b. Perfusion solutions and equipment

The solutions for the perfusions were made from the following mixtures: Stock A**:** 0.2M Sodium dihydrogen orthophosphate (MW 137) (Merc Millipore: NaH2PO4 Cas-No:10049-215) and Stock B: 0.2M Disodium hydrogen orthophosphate (MW 142) (Merc Millipore: NaH2PO4 Cas-No: 7558-79-4). The phosphate buffer (PB) solution was: PH 7.2 = 140ml stock A & 360ml stock B & 500ml dH_2O for 1 litre of PB. Phosphate buffered saline (PBS) was made by PB & 8.87g/litre sodium chloride molecule (NaCl) at a molecular weight (MW) of

(MW 58.44). The 4% PFA: 40gPFA (Merc-Millipore 104005 Cas-No: 30525-89-4) was mixed into 100ml PB, was placed on a heated and magnetized thermo-plate (Gimral3 Thermolyne) and stirred until the PFA was totally dissolved without any powder visible. The PFA was then strained until fully clear and was placed in -4% fridge ready for use on the day it was produced. The 20% Sucrose: 20g sucrose #033 -5KG Lot M 1371Co71 (MW 342.3) in 100ml PB. All measurements for solutions were made on a Mettler PM4800 Delta Range scale and stirred until fully dissolved.

The following peristaltic setup was utilized for the perfusions 1/ a Master Flex L/S Drive 1-100 RPM peristaltic pump 2/ Pump Head/Easy Load II, 3/ Mounting Hardware with 2 Heads (to piggyback two heads on one pump), 4/ a foot-pump lifted to the bench so it could pumped by hand, 5/ Stopcock, 3-way, female to male.

Appendix L. Neuronal cell counts mean and SD.

Table 36 Immunohistochemistry Cell Counts

Average counts of cells exhibiting florescent Fos-like activity and neurotransmitter florescent labelling of NPY and α-MSH in mouse brain sections.

Groups (n=5)	c-Fos		NPY		α-MSH	
ARC	Mean	SD	Mean	SD	Mean	SD
SNO-100CFE -2DG	88.2	± 19.18	51	± 37.51	28.2	± 10.47
SNO-100CFE-SAL	118.8	± 28.41	77.2	± 43.82	16.2	± 15.99
SNO-PLAC-2DG	49.6	± 16.68	21	± 15.93	16.8	± 12.11
SNO-PLAC-SAL	51	± 15.84	24	± 12.94	3.8	± 3.11
WT-100CFE-2DG	146.2	± 43.56	109.2	± 42.84	20	± 14.07
WT-100CFE-SAL	138.6	± 24.13	62.6	± 33.9	30.8	± 24.24
WT-PLAC-2DG	218.4	± 55.38	159.8	± 65.53	20.8	± 11.88
WT-PLAC-SAL	128.6	± 40.12	98.6	± 47.41	17.6	± 9.15
PVN						
SNO-100CFE -2DG	78	± 16.29	37.6	± 23.73	6.4	± 8.9
SNO-100CFE-SAL	79.6	± 29.19	53.8	± 28.08	9.2	± 7.05
SNO-PLAC-2DG	51	± 15.84	24	± 12.94	3.6	± 3.21
SNO-PLAC-SAL	97.2	± 43.05	42.8	± 27.66	1.75	± 2.19
WT-100CFE-2DG	97.4	± 39.03	63.2	± 27.6	2.6	± 4.16
WT-100CFE-SAL	102.8	± 27.87	54.6	± 13.87	5	± 3.87
WT-PLAC-2DG	138.2	± 49.17	76	± 18.07	7.8	± 6.42
WT-PLAC-SAL	107.8	± 23.49	48.4	± 24.47	3.6	± 3.13
MnPO						
SNO-100CFE -2DG	73	± 28.43	21	± 22.8	3.6	± 4.15
SNO-100CFE-SAL	84.2	± 15.02	47.2	± 16.02	7.4	± 7.13
SNO-PLAC-2DG	22	± 3.81	12.4	± 5.22	0.8	± 1.3
SNO-PLAC-SAL	63.8	± 17.38	35.6	± 10.38	0.8	± 0.89
WT-100CFE-2DG	48.4	± 15.47	34.8	± 15.16	0.4	± 0.89
WT-100CFE-SAL	110.4	± 42.82	35.8	± 30.11	0.8	± 1.09
WT-PLAC-2DG	84.8	± 27.34	63.4	± 37.54	3.2	± 4.08
WT-PLAC-SAL	110.8	± 29.35	51	± 33.33	9.8	± 11.71

Table 36. Mean and SD - standard deviation results of immunohistochemistry cell counts c-Fos:- Fos like early gene expression, NPY: - neuropeptide –Y and α-MSH: - alpha - Melanocyte-stimulating hormone, in brain slices from three regions of interest; the ARC: - arcuate nucleus, PVN, paraventricular nucleus and MnPO: - median preoptic nucleus, as representative sections from the hypothalamus and lamina terminalis in brain slices from two mouse strains: SNO: - Garvan *Snord116del* and WT: - C57BL/6 wild type, with (n=5) five animals in each group. All animals were ingesting either chronic treatment 100CFE: - *Caralluma fimbriata* extract, at 100mg/kg/d or PLAC: – placebo of maltodextrin/cabbage leaf with appetite signalling reagents, 2DG: - 2-deoxy-glucose compared to the control of SAL: - saline.

Appendix M. Mia's story and history

A single case study

Study one has been instigated by anecdotal evidence from a single case study (Griggs, 2010). A diagnostic adjustment was made from suspected Cerebral Palsy to Developmental delay and then to PWS at eighteen months of age. This was due to weight gain, shortness in stature and hyperphagic behavioural indicators. The diagnosis led to researching natural remedies and the eventual use of CFE as an intervention treatment. Careful dosage was considered by direct communication with the manufacturers and administration of a half a capsule (250mg) with water each morning. This started at 29 months of age. Eventually due to taste the 'Slimaluma' CFE supplement was given daily within tropical juice (to cover the organic taste) and the dose was raised in 250mg increments. The raising of dose was mainly when the appetite markers characteristically moved towards hyperphagia again. The eventual dose for weight, at age nine, necessitated capsules at a dose of 1250mg. This amount continues to sustain successful appetite behaviour.

In 2010, Mia ceased taking the extract for 6 days. Obvious changes to behaviour were recorded, including, hunger, crying, tantrums, stuttering, OCD and constant repeated food related questions. On the resumption of the extract, these indicators disappeared after three days.

In 2011 after 4 years and six months' administration of CFE, M. was given blood tests to check for any undue toxicity or effect due to CFE administration: B12 folate, iron studies, vitamin d, kidney function, liver function tests, blood count, IGF and thyroid function and HbA1C - measure of hyperglycaemia were all within normal range.

I label Mia as inspirational. She made us stand up whilst we were trying to teach her to do exactly same thing. Mia's early fight with her body led us to become advocates. Her hunger led to a momentous turning point and a search for an intervention with the capacity to inspire independence in some with PWS. Mia made me become who I am today. The dilemmas we have negotiated through our Multiple Initiative Approach (MIA), over eleven years are all part of the individualized package in PWS: vision impairment – Mia had her eyes patched for years, stuttering, deteriorating teeth -silver crowns, scoliosis - 57°, Mia has worn a plaster jacket for

months and has lived in a Scolicare brace daily, anxiety - Mia has been ingesting *fluoxetine* for skin picking since she was nine, early and incomplete puberty – more to come?, incapacity to generalize, repetitive questioning, a fear of falling over - exercise program to help her hypotonicity, lying and making up stories, being bullied, broken bones, immaturity (more infantile than others) and of course a concentration on food - CFE nine years. Mia has had five operations with the accompanying PWS operative safety requirements; endocrine tests for CAI and associated blood tests. Mia has had three sleep studies, been deemed allergic to growth hormone after months of needles in her thighs, she has also experienced years of speech therapy, physiotherapy and occupational therapy. Yet at 11 years of age Mia is 32 kilo and 129 cm, capable of living in a non-restricted environment, on her way to secondary school and she enjoys her ballet dancing weekly even though it's difficult. Mia rarely complains. This is one reason for her success and the rest is her family, the continued observation, intervention and CFE.

We believed in CFE from the moment we saw Mia reach past food for a doll when she was two years of age. It wasn't just obesity or prejudice, or the constant hard work of surveillance, that worried us. It was what they represented for Mia and our family; freedom, independence! We wanted developmental gains for Mia and since the first weeks of Mia ingesting CFE, her "Yuckits" – independence has become more than a hope. Soon we may even say an option. Real independence without supervision, like we take for granted every-day. No CFE is not accumulative in action… so unfortunately there is work ahead on dose, as this may become a problem.

In 2010, Mia ceased taking the extract for six days. Obvious changes to behaviour were recorded, including, hunger, crying, tantrums, stuttering, OCD and constant repeated food related questions. On the resumption of the extract, these indicators disappeared after three days. In 2011 after years of administration, Mia was given blood tests to check for any undue toxicity or effect, due to CFE. Many markers were tested: B12 folate, iron studies, vitamin d, kidney function, liver function tests, blood count, IGF and thyroid function and HbA1C – a measure of hyperglycaemia. These were all within normal range. So too Mia completed a test for central adrenal insufficiency (CAI) in 2013, before going through her spinal operations and this was fine. These are good signs but there is more work ahead.

After >8 years' administration of CFE, Mia continues to do well, (1250mg/per day). We are on the way to independence and thanks to this research and so may be others. Thanks to all those who have chosen to give it a go. Luckily imbedded in the nature of many families whose children have disabilities; seems to be a determination to fight the list of global difficulties presented to them. Disabilities are individual and complex and life is not only restricted by typical hurdles but unpredictable ones with unpredictable outcomes. Most parents see these documented in the list of appointments with many unsatisfactorily answered questions. Yet we advocate to highlight these difficulties in a positive path of rebellion and research, which often lands us with hard work and eventual answers and sometimes treasures.

Completed in 2016

With Gratitude