Examining the role of the Prader-Willi syndrome critical interval in psychiatric illness and cognition

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By

Simona Kirilova Zahova

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SECTION 1: CANDIDATE'S DETAILS						
Full name	Simona Zahova					
Student number	C1230848					
School	Medicine					
Degree	PhD					
Full title of thesis	*Examining the role of the Prader-Willi syndrome critical interval in psychiatric illness and cognition*					
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Summary

Prader-Willi Syndrome (PWS) is a neurodevelopmental disorder caused by loss of function mutations on chromosome 15q11.2-q13. It is characterized by hypotonia and hyperphagia, as well as impaired cognition and attention, maladaptive behaviour and increased prevalence of psychiatric illness including anxiety, depression, and psychosis. The hyperphagia and hypotonia observed in individuals with PWS are attributed mainly to the *SNORD116* and *IPW* non-coding RNAs on chromosome 15, which are collectively known as the Prader-Willi syndrome critical interval (PWS-cr). However, the genetics underlying the cognitive and psychiatric disorders in PWS are still not well understood. The aim of this study is to assess the role, if any, of PWS-cr in cognition and psychiatric illness in three approaches: by investigating behaviours of relevance in a mouse model carrying a deletion of PWS-cr (Chapters 2, 3), by examining the transcriptomic effects of the deletion on the neonatal mouse brain (Chapter 4) and by analysing the effect of genetic variation in the PWS-cr interval in humans on phenotypes of relevance (Chapter 5).

Previously, endophenotypes of relevance to ADHD and psychotic illness have been reported in a "full" mouse model of PWS (PWS-IC), in which expression of the entire PWS locus including PWS-cr is dysregulated. In comparison, the PWS-cr mouse model exhibited no behaviours of relevance to ADHD and psychotic illness, but exhibited a behaviour that could be indicative of depression.

In order to investigate the molecular factors driving the behavioural phenotypes of PWS-IC and PWS-cr mouse models, RNA-sequencing study of neonatal brain of both models was performed. The results revealed transcriptomic changes relevant to psychosis in the PWS-IC mouse model, which were absent in the PWS-cr model. However, the PWS-cr mouse brain had increased expression of *Necdin* and differential isoform usage of *Dyrk3*, which are both linked with cognition and learning.

Since the behavioural and transcriptomic studies provided evidence that PWS-cr might play a role in depression and cognition, the role of the interval in depression and cognition was examined by investigating whether genetic variants in the interval are associated with phenotypes of relevance. The results showed no evidence of genetic variants within PWS-cr having a role in depression, but gave an indication that PWS-cr might be involved in rate of processing visual figures.

In conclusion, results from this study suggest that PWS-cr might play a subtle role in aspects of cognition, but its deletion does not induce most of the behavioural and molecular changes observed in individuals with PWS or in the PWS-IC mouse model.

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List of Abbreviations

5-CSRTT	5-choice serial reaction time task
ADD	Attention deficit disorder
ADHD	Attention deficit hyperactivity disorder
ASD	Autism spectrum disorder
ASR	Acoustic startle response
BMI	Body mass index
CNV	Copy number variation
delPWS	PWS genetic subtype: deletion spanning the PEGs
DMR	Differentially methylated region
dNTP	Deoxynucleoside triphosphate
DST	Digit span test
EPM	Elevated plus maze
FIT	Fluid intelligence test
glm	generalized linear model
GWAS	Genome-wide association study
IC	Imprinting centre
IPW	Imprinted in Prader-Willi
ITI	Inter-trial interval at the 5-CSRTT
lm	linear model
LMA	Locomotor activity
lncRNA	Long non-coding RNA
MDD	Major depressive disorder
MEG	Maternally expressed gene
mUPD15	PWS genetic subtype: maternal uniparental disomy of chromosome 15
OF	Open field
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEG	Paternally expressed gene
PMT	Pairs matching test
PPI	Pre-pulse inhibition

PRS	Polygenic risk score
PWS	Prader-Willi syndrome
PWS-cr	Prader-Willi syndrome critical interval/PWS-cr deletion mouse model
PWS-IC	Prader-Willi syndrome imprinting centre deletion mouse model
RTT	Reaction time test
SDST	Symbol digit substitution test
SEM	Standard error of the mean
SNORD116	Small nucleolar RNA, C/D box 116
snoRNA	Small nucleolar RNA
SNP	Single nucleotide polymorphism
TMTA/B	Trail making tests A and B

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Chapter 1. General introduction

Prader-Willi Syndrome (PWS) is a rare neurodevelopmental disorder that affects 1:10 000 to 1:25 000 people (Burd et al. 1990; Butler 1990; Åkefeldt et al. 1991). It is caused by loss of function mutations on a locus of imprinted genes on chromosome 15q11.2-q13. Among these genes are two non-coding RNAs collectively known as the PWS critical interval (PWS-cr). The research described here aims to examine whether PWS-cr has a role in psychiatric and cognitive phenotypes.

1.1 Overview of Prader-Willi syndrome

PWS is characterized by hypotonia and slow growth rate in infancy followed by severe hyperphagia and obesity throughout childhood and adulthood, as well as mild to moderate learning disability, psychiatric illness and a range of different maladaptive behaviours of relevance to autism spectrum disorder (ASD) (Cassidy et al. 2011; Angulo et al. 2015). Furthermore, individuals with PWS between the ages of 5 and 56 exhibit an elevated mortality rate of 3% compared to 0.14% that is observed in the general population (Whittington et al. 2001).

This disorder significantly lowers quality of life and wellbeing of individuals directly affected by it in physical, social and mental aspects, compared to typically developing peers and peers with obesity (Caliandro et al. 2007; Wilson et al. 2016). Individuals with PWS experience emotional turmoil, elevated anxiety, depression and self-injurious behaviour (Bouras et al. 1998; Gross-Tsur et al. 2001; Dykens and Shah 2003). Furthermore, studies have demonstrated that the quality of life of parents, siblings and care-givers is also impaired (Mazaheri et al. 2013; Wilson et al. 2016; Mao et al. 2019). Parents of individuals with PWS have been reported to exhibit elevated anxiety, somatisation, and obsessive-compulsions (Skokauskas et al. 2012), while a study on siblings showed that up to 92% of them exhibited symptoms of post-traumatic stress disorder (Mazaheri et al. 2013). The factors contributing to reduction in wellbeing include the stress around controlling the hyperphagic behaviour and the resulting obesity, but also the behavioural problems and psychiatric illness, which tend to worsen with age, and the deterioration of which has also been linked to decreased quality of life in parents and care-givers (Ihara et al. 2014).

Fortunately, therapies that target the health outcomes related to height, appetite and weight have seen a very promising development in the last few decades. Growth hormone therapy in particular, has been effective in inducing a positive effect on body composition and physical health in children and adults, and even on some aspects of

cognitive function (Höybye et al. 2005; Sanchez-Ortiga et al. 2012; Deal et al. 2013; Damen et al. 2020; Donze et al. 2020). However, when it comes to the psychiatric and behavioural problems, there's still a dearth of targeted therapies (Bonot et al. 2016). One of the reasons for the scarcity of psychotropic therapies is the lack of research of the molecular and neuronal mechanisms responsible for the psychiatric profile of individuals with PWS. A foundational step in researching the mechanisms behind these phenotypes is establishing which genes of the PWS locus contribute to them. Furthermore, investigating the genetics of psychiatric illness in PWS is relevant not just to the treatment of this rare disorder, but potentially also to our understanding of behavioural and psychiatric disorders such as autism, anxiety, depression, bipolar disorder, schizophrenia, and general psychosis.

1.1.1 Genetics of Prader-Willi syndrome

PWS is caused by loss of function mutations affecting the expression of a cluster of genes on 15q11.2-q13. The genes in this cluster are imprinted, which means that they are expressed only from one parental copy of the chromosome, and suppressed on the other. The 15q11.2-q13 region contains some genes that are expressed only from the paternally inherited copy of the chromosome (aka paternally expressed genes - PEGs) including the protein-coding *MKRN3, MAGEL2, NECDIN, NPAP1,* and *SNURF-SNRPN,* and a cluster of non-coding RNAs (Figure 1.1). 15q11.2-q13 also includes two maternally expressed protein-coding genes (MEGs) (*UBE3A* and *ATP10A).* Loss of expression of the PEGs leads to PWS, while the loss of expression of MEGs leads to another neurodevelopmental disorder – Angelman syndrome (Clayton-Smith and Laan 2003; Angulo et al. 2015).

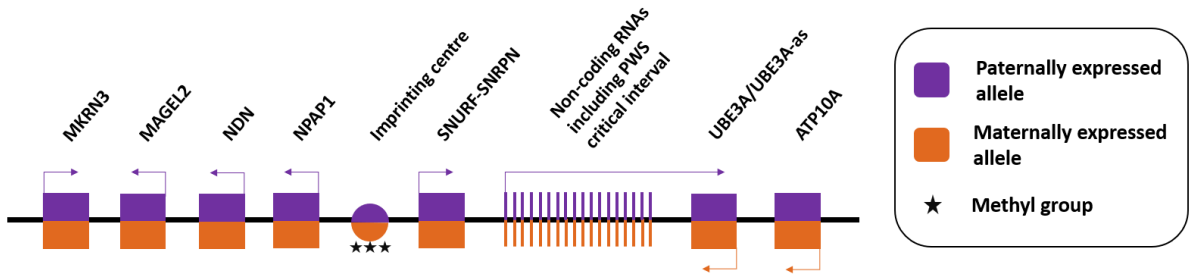


Figure 1.1 The Prader-Willi locus. The 15q11.2-q13 imprinted region contains five paternally expressed protein-coding genes and a cluster of paternally expressed non-coding RNAs. The locus also includes two maternally expressed protein-coding genes. The imprinting of the locus is established by DNA methylation on the maternal copy of the chromosome, in a regulatory region upstream of the paternally expressed *SNURF-SNRPN* allele. This regulatory region is known as the imprinting centre.

1.1.2 Mechanism of imprinting of 15q11.2-q13

The imprinting of the PWS locus is established by differential methylation of the maternally inherited copy of chromosome 15, at the 5' end of the bicistronic SNURF-SNRPN gene. Whether the depositing of these methyl groups takes place during gametogenesis or fertilisation is a matter of contention in literature (Glenn et al. 1996; El-Maarri et al. 2001; Geuns et al. 2003; Horsthemke 2008). This differentially methylated region (DMR) is known as the imprinting centre (IC) of the PWS cluster (Figure 1.1). On the maternally inherited copy of chromosome 15, the DNA methylation of the IC is combined with methylation of histones H3 on Lys9 and H4 on Lys20, which collectively serve to repress the transcription of the PEGs (Xin et al. 2001; Fournier et al. 2002).

Furthermore, the IC regulates the transcription of a long non-coding RNA, which is antisense to the maternally expressed gene *UBE3A* (*UBE3A-as*). *UBE3A-as* binds to the transcript of *UBE3A* leading to a double-stranded RNA formation, which triggers the Dicer digestion mechanism, and thus suppresses the expression of the paternal copy of *UBE3A* (Chamberlain 2001; Runte et al. 2001). Since the methylation of DNA and chromatin on the maternally inherited copy of chromosome 15 suppress all the genes that the IC promotes, *UBE3A-as* is not expressed there to silence the maternally inherited copy of *UBE3A*.

1.1.3 Genetic subtypes of Prader-Willi syndrome

Individuals with PWS can be roughly divided into two groups based on distinct genetic subtypes. The first group consists of those that carry a paternally-derived deletion spanning the genes of the 15q11.2-q13 locus (delPWS). delPWS is the more prevalent genetic subtype, observed in approximately 75% of people with PWS.

A smaller subset of people with PWS (~25%) carry a maternal uniparental disomy of chromosome 15 (mUPD15). In addition to complete loss of expression of all PEGs, mUPD15 leads to an overexpression of the two MEGs in the 15q11-q13 imprinted interval. A similar gene expression profile is also observed in the much rarer IC deletion subtype (<1%), which leads to loss of imprinting of the whole 15q11-q13 region and results in loss of expression of the PEGs and overexpression of the MEGs (Whittington et al. 2007).

Crucially, these distinct genetic subtypes have been shown to lead to distinct phenotypical outcomes in terms of physical health, cognition and psychiatric illness (Roof et al. 2000; Boer et al. 2002; Verhoeven et al. 2003; Manzardo et al. 2018).

1.1.4 Impairment of the endocrine system

The main features of PWS are caused predominantly by dysfunctions of the endocrine system. Brain imaging studies have shown that individuals with PWS exhibit an unsual bright spotting in the posterior pituitary and altered functional hypothalamic connectivity with lateral occipital complexes in both hemispheres (van Nieuwpoort et al. 2011; Lukoshe et al. 2017b). Furthermore, studies on mouse models have shown that a few of the PEGs from the PWS locus, namely *Snord116, Necdin* and *Magel2,* play a role in hypothalamic development, morphology and function (Muscatelli et al. 2000; Pravdivyi et al. 2015; Polex-Wolf et al. 2018). These alterations in the pituitary and the hypothalamus affect endocrine function and secretion of hormones. Consequently, these alterations are thought to contribute to the PWS phenotypes related to growth, metabolism, hunger, sexual development and sleep.

Individuals with PWS exhibit postnatal growth retardation predominantly caused by deficiency in growth hormone secretion, which continues through puberty and results in a short stature in adulthood (Grosso et al. 1998; Bertella et al. 2007; Grugni et al. 2016). The growth retardation is further exacerbated in the first year of life due the presense of neonatal hypotonia, aka “floppy infant syndrome,” which is observed in 97% of individuals diagnosed with PWS (Gunay-Aygun et al. 2001). The hypotonia results in poor sucking reflex, and consequently in feeding difficulties and reduced postnatal weight gain, as well as diminished tendon reflexes, reduced crying and lethargy (Miller et al. 1999; Trifirò et al. 2003; Denizot et al. 2004). Notably, PWS is responsible for approximately 10% of neonatal hypotonia (Tuysuz et al. 2014). It persists into adulthood, affects balance and gait pattern, and overall mobility (Cimolin et al. 2011; Galli et al. 2011).

The initial postnatal feeding difficulties fade out after the first year of life, and around the age of 3-5 years most individuals with PWS begin exhibiting an increased interest in food, which then develops into full-blown hyperphagia by the age of 8 (Miller et al. 2011). In PWS, hyperphagia is caused by disruption of hypothalamic pathways that regulate satiety (Swaab et al. 1995; Shapira et al. 2005; Hinton et al. 2006; Cassidy et al. 2011), resulting in elevated levels of circulating ghrelin hormone (Cummings et al. 2002; Haqq et al. 2008), an orexigenic stimulant of appetite (Schmid et al. 2005; Levin et al. 2006). Obesity is observed in most individuals with PWS due to the deficits in satiety function as well as mobility difficulties, although obesity can be reduced if eating is carefully managed. In addition to hyperphagia, obesity may also be caused

by alterations in metabolism, including unstable blood glucose levels, increased fat storage, low muscle tone, and thyroid hormone deficiencies, which result from and also lead to reduced energy expenditure (Mogul et al. 2008; Sode-Carlsen et al. 2010; Castner et al. 2014; Khan et al. 2018).

Endocrine dysfunction also leads to increased prevalence of pubertal retardation or arrest and genital abnormalities. Studies show that the majority of individuals with PWS have hypogonadism and infertility, as well as hypoplasia or complete absence of testes or labia minora, likely due to low levels of luteinizing hormone-releasing hormone (LHRH), which is associated with decreased sex hormone levels and retardation of puberty (Nagai and Mori 1999; Crinò et al. 2003; Vogels et al. 2008; Radicioni et al. 2012). Cases of precocious puberty are also increased among people with PWS compared to the general population (Crinò et al. 2003). Exome studies of patients diagnosed with idiopathic central precocious puberty (ICPP) found missense variants and frameshift mutations in *MKRN3* that lead to pathogenic protein changes. The same studies found no pathogenic variants of the expected *KISS1, KISS1R, LIN28, GNHR, GNRHR, TACR3* and *TAC3*, which suggests that the PWS PEG *MKRN3* might be one of the leading causes of sexual maturation pathologies (Abreu et al. 2013; Ortiz-Cabrera et al. 2017), and its loss of expression in individuals with PWS explains the sexual maturation delays that are observed among them.

The alterations in hypothalamic function in PWS also result in disturbance of circadian rhythms. Individuals with PWS exhibit abnormal sleeping patterns, daytime hypersomnolence and an overall disturbance of sleep architecture, including ratio of rapid-eye-movement (REM) to non-rapid-eye-movement (NREM) episode cycles (Hertz et al. 1993; Priano et al. 2006; De Cock et al. 2011).

1.1.5 Cognition

Individuals with PWS exhibit mild-to-moderate learning disability (Gross-Tsur et al. 2001; Whittington et al. 2004; Reddy and Pfeiffer 2007; Yang et al. 2013). A cognition study of 18 patients with PWS showed signs of learning disability in all the examined individuals, presenting as dyscalculia, dysgraphia and in 75% of patients —dyslexia (Gross-Tsur et al. 2001). The average IQ score on the Wechsler Full-Scale was 73.7, falling approximately one standard deviation below the normal range. Interestingly, 15 of these individuals showed a high discrepancy of 15 or more points between verbal and performance IQ in reverse patterns that did not correlate with genetic subtype.

Although Gross-Tsur et al. (2001) didn't discover a reliable trend of differences in cognitive performance between individuals with delPWS and those with mUPD15, several other studies that have reported distinct cognitive phenotypes linked to the two genetic subtypes (Cassidy et al. 1997). In a study of 38 individuals with PWS, Roof et al. (2000) reported a significant difference between the cognition of the two groups. Subjects with mUPD15 exhibited significantly higher verbal IQ (Wechsler scale), with an over 9 point difference, which pushed the group up to the threshold for 'mild mental retardation' as defined by the American Psychological Association, whereas the delPWS group's score was well into the range for this classification. Furthermore, the delPWS group scored significantly higher than the mUPD15 group in the Object Assembly test performed by the same study, which measures visuo-perceptual skills. This led Roof et al (2000) to suggest that the mUPD15 genetic subtype might result in decreased ability to recognize shapes that require use of stereoscopic vision. This proposal was affirmed a few years later by a study of 92 individuals with PWS, which replicated the distinct cognitive phenotypes linked to delPWS and mUPD15 genotypes, and further discovered impaired coding abilities (indicative of visuo-motor abilities) in individuals with mUPD15, but not in those with delPWS (Whittington et al. 2004).

Studies prompted by anecdotal reports of high aptitude for jigsaw puzzles in children with PWS confirmed that these children outperform not only age-matched peers with intellectual disability, but also those with average IQs by placing a significantly higher number of pieces in a set time than those placed by their control groups (Dykens 2002; Rosner et al. 2004; Whittington et al. 2004). Interestingly, this skill was only observed in individuals with delPWS and not in those with mUPD15, which ties to Roof et al's (2000) report of higher object assembly and shape recognition performance in the former group compared to the latter.

Furthermore, individuals with PWS exhibit an increased prevalence of phenotypes typical of attention-deficit disorders (ADD/ADHD) (Gross-Tsur et al. 2001; Wigren and Hansen 2005). In a cognition study of 18 individuals with PWS, 13 were previously diagnosed with ADHD and out of those, eight scored within the pathologic range (>98 percentile) on the Child Behavioural Checklist attention scale (Gross-Tsur et al. 2001). A study by Wigren and Hansen (2005) discovered not only clinically significant scores on a broad ADHD index, but also linked those scores to increased severity of maladaptive behaviours and conduct problems.

Overall, studies of cognition in PWS reveal an interesting phenotypical dichotomy between the genetic subtypes, with the delPWS individuals exhibiting a more severe form of learning disability compared to their peers with mUPD15, while at the same time exhibiting an aptitude for visuo-perceptual skills that is not present in those with mUPD15. One possible explanation for the phenotypical outcomes of the two genetic subtypes is that the loss of expression of the PEGs is responsible for learning disability and enhanced visuo-perceptual skills in both groups, and that the overexpression of the MEGs in individuals with mUPD15 compensates for that. This is further supported by mouse model studies which show that the deletion of the PEGs *Snord116* and *Necdin* might have a role in impairing some types of cognition while enhancing other (Muscatelli et al. 2000; Adhikari et al. 2019). Currently, there are very few studies that have examined ADD/ADHD in individuals with PWS, and none that show whether or how it is affected by genetics.

1.1.6 Psychiatric illness

Psychiatric illness is highly prevalent in individuals with PWS, who exhibit episodes of anxiety, depression and/or psychosis occurring as one-time events or in a recurring fashion (Boer et al. 2002; Dykens and Shah 2003; Thuilleaux et al. 2018b). The Psychological General Well-Being Index and Birleson's Depression Self-rating Scale show that people with PWS score highly for depression, which manifests as lack of energy, lethargic behaviour, negativity and social withdrawal (Akefeldt and Gillberg 1999; Bertella et al. 2007; Skokauskas et al. 2012). Furthermore, a study of psychiatric illnesses in 94 individuals with PWS showed that over 25% have at some point been prescribed antidepressant or mood-stabilizing medication (Soni et al. 2007). Individuals with PWS also exhibit a higher prevalence of anxiety than their neurotypical peers and their peers with intellectual disability (Wigren and Hansen 2005; Reddy and Pfeiffer 2007).

Psychotic symptoms have also been well documented in individuals with PWS, including paranoid ideation, delusional thinking and auditory and visual hallucination (Kollrack and Wolff 1966; Bray et al. 1983; Whitman et al. 1987; Bartolucci and Younger 1994; Bouras et al. 1998). These symptoms are most commonly diagnosed as cycloid psychosis and schizophrenia-spectrum disorder, although consensus for the diagnosis is still lacking, since neither disorder perfectly matches the typical psychotic profile of PWS (Bouras et al. 1998; Verhoeven et al. 2003; Soni et al. 2008). Among those with psychosis, a large proportion fulfil the criteria for bipolar affective

disorder, including symptoms of hypomania; expansive moods, racing thoughts, over-activity and a decreased need for sleep (Soni et al. 2008).

As with cognition, studies show two distinct psychiatric profiles linked with the two different PWS genetic subtypes. Depression in PWS is broadly divided it into two types, based on whether it's accompanied by psychotic symptoms or not (Soni et al. 2008). A study of 102 individuals with PWS reported that depression without psychosis was the most common form of psychiatric illness in the delPWS group, whereas in the mUPD15 group the most common form of psychiatric illness was a form of psychosis with depressive components (Sinnema et al. 2011). Regardless, the symptoms of depression itself do not differ between these two psychiatric profiles (Soni et al. 2008), which suggests that the depressive symptoms might be caused by the loss of expression of the PEGs in the PWS region, while the psychotic illness might be caused by the overexpression of the MEGs. The role of the MEGs in psychosis is further solidified by a study that demonstrated that maternally inherited duplications spanning the MEGs are a risk factor for schizophrenia (Isles et al. 2016). Although psychotic symptoms are also linked to the delPWS genetic subtype, it is 2-4 fold higher in individuals with mUPD15 (Boer et al. 2002; Soni et al. 2007; Sinnema et al. 2011). Furthermore, a mouse model carrying a deletion of the imprinting centre (PWS-IC), which replicates the PWS region gene expression of the mUPD15 subtype, exhibits behaviours of relevance to psychosis (Relkovic et al. 2010).

One of the suggested roots of psychiatric illness in PWS is dysfunction of the serotonergic system, which is linked to both anxiety and depression (Iwamoto and Kato 2003; You et al. 2005; Chang et al. 2017). A study on children with PWS showed an increased concentration of a serotonin metabolite in cerebrospinal fluid, which suggests an increased serotonin turnover (Akefeldt et al. 1998). Furthermore, antipsychotic drugs with antagonistic activity over the 5HT-serotonin receptor have shown a positive effect on treatment of psychosis and disruptive behaviours (Durst et al. 2000). Notably, the PEGs *SNORD115, MAGEL2,* and *NECDIN* have been linked to the regulation of the serotonergic system (Kishore and Stamm 2006b; Zanella et al. 2008; Doe et al. 2009; Mercer et al. 2009).

From the MEGs, *UBE3A* is considered to be the main cause of psychiatric illness. A case of a family carrying a duplication that spans only the *UBE3A* gene exhibited a whole spectrum of cognitive and psychiatric phenotypes relevant to PWS, including learning disability, schizophrenia and behaviours typical of autism (Noor et al. 2015). The findings from this case were further supported by a recent genomic study that

found a link between variation in *UBE3A* and paranoid ideation and delusions (Salminen et al. 2019).

1.1.7 Behaviour

Studies report a range of maladaptive behaviours in individuals with PWS, including poor impulse control, tantrum-throwing, skin-picking, and propensity for compulsive and inflexible behaviour (Dykens et al. 1992; Dykens 2004; Rosner et al. 2004; Kim et al. 2005; Wigren and Hansen 2005; Manzardo et al. 2018). An examination of maladaptive behaviours in 21 adolescents and adults with PWS using the Vineland Adaptive Behaviour Scales and Achenbach's Child Behaviour Checklist also showed issues with socialization, worse than those observed in individuals with Williams syndrome and Down syndrome (Dykens et al. 1992; Rosner et al. 2004).

Individuals with PWS exhibit compulsive traits, not related to cleanliness, but to ritualistic behaviour that's associated with childhood compulsive behaviours (Wigren and Hansen 2003,2005). Insistence on sameness and inflexibility with daily routine were studied in 50 people with PWS (aged 5-18 years) with a control group of neurotypical 4-year-old children. Individuals with PWS not only scored higher than 4-year-olds on the Childhood Routines Inventory for intense compulsive behaviour, but also those behaviours did not reduce with age (Wigren and Hansen 2003) unlike what is observed in typically developing children (Bolton 1996; Zohar and Bruno 1997).

The most prominent example of compulsive behaviour in PWS is skin-picking, which is a form of self-injury that is observed in nearly 50-100% of individuals with PWS, depending on the criteria of reporting (Gross-Tsur et al. 2001; Dykens 2014; Whittington and Holland 2020). A study on self-injurious behaviour in adults with intellectual disability showed a 10-20 fold increased prevalence of skin picking in individuals with PWS compared to their peers with intellectual disability (Cooper et al. 2009).

Skin picking has been linked to impairments in the serotonergic system (Denys et al. 2003; Dufour et al. 2010; Grant et al. 2012), which is supported by studies on SSRI treatment in individuals with PWS, which showed a reduction of this behaviour after treatment (Warnock and Kestenbaum 1992; Hellings and Warnock 1994). On a genetic level skin picking has been linked to the loss of expression of *NECDIN*, since a mouse carrying a deletion of the *Necdin* gene is the only PWS mouse model that has replicated this behaviour (Muscatelli et al. 2000), and since *Necdin* has been linked to the serotonergic system (Zanella et al. 2008). However, an individual with a microdeletion spanning *NECDIN* did not exhibit skin picking (Kanber et al. 2008),

while a couple of individuals with deletions spanning the non-coding RNAs of the PWS locus exhibited this behaviour (Sahoo et al. 2008; Duker et al. 2010), which suggests that skin picking might be linked to the hypothalamic and striatal changes caused by loss of expression of the PWS non-coding RNAs.

It has to be noted that individuals carrying deletions of the PWS non-coding RNAs also exhibit the core features of PWS including hyperphagia, and affective psychiatric illness, which suggests that skin-picking might be a downstream effect of these phenotypes rather than a direct result of the deletions. Although skin-picking is seen as compulsive-like behaviour, medication targeting compulsive behaviour has been shown as ineffective (Warnock and Kestenbaum 1992; Benjamin and Buot-Smith 1993), and furthermore, a statistical clustering study of the maladaptive patterns of behaviour in PWS discovered that skin-picking does not cluster together with other phenotypes of obsessive-compulsive disorder (Pignatti et al. 2013). Overall, evidence suggests that at least some of the behavioural problems in PWS are caused by the cognitive and psychiatric phenotypes.

For example, a study of 240 individuals with PWS showed that BMI is in a reverse correlation with skin-picking, since being on a more strict diet to maintain a healthy weight led to increased stress and anxiety (Dykens 2004) and individuals with PWS themselves attribute this behaviour to nervousness (Didden et al. 2008; Morgan et al. 2010). Skin-picking in PWS has also been linked with ADHD (Cooper et al. 2009), as have disruptive behaviours of tantrum-throwing (Woodcock et al. 2009). A study by Woodcock et al. (2011) of 4 individuals with PWS linked temper outbursts with deficiencies in attention switching and insistence on sameness. This falls in line with a series of studies that reported a high comorbidity of ADHD/ADD with conduct problems (Lazar and Frank 1998; Wu et al. 2002; Sukhodolsky et al. 2003).

Collectively, the phenotypes of inflexible and compulsive behaviour, issues with socialisation and disruptive temper observed in PWS overlap with those of ASD. A systematic review of studies of ASD in PWS revealed that about a quarter of individuals with PWS also have a diagnosis of ASD (Veltman et al. 2005). Behavioural problems observed in ASD such as increased irritability and aggression, attentional lapses and insistence on sameness have been linked with high anxiety (Gotham et al. 2013)

Notably, both compulsive-like behaviour and ADHD have been associated with abnormalities in the prefrontal-striatal circuitry and impaired executive function (Evans et al. 2004; Snyder et al. 2014). These behaviour along with neuroimaging

studies showing diminished activity in prefrontal cortex areas add to growing evidence of Prader-Willi Syndrome being linked to executive dysfunction (Holsen et al. 2012; Zhang et al. 2013). A study by Chevalère et al. (2015) of 17 individuals with PWS with 17 neurotypical matched controls showed signs of executive dysfunction in the former group even after controlling for IQ. Interestingly, a MRI study by Holsen (2012) of 14 individuals with PWS along with 14 matched controls with obesity showed that hypoactivity of the prefrontal circuitry is particularly observed after feeding. This phenomenon was observed in cortical areas responsible for inhibitory control and decision making.

Overall, the cognitive, psychiatric and behaviour phenotypes of PWS are tightly interlinked and it is possible that the presentation of each individual symptom is affected by the overall phenotype of the syndrome.

1.2 The role of the Prader-Willi syndrome critical interval

Among the non-coding RNA PEGs of the PWS-region, two are known as the PWS critical interval (PWS-cr), because their deletion is thought to be responsible for the main phenotypes of PWS. There are currently six reported cases of people carrying a micro-deletion spanning the PWS-cr interval (Figure 1.2) (Butler et al. 1996; Sahoo et al. 2008; de Smith et al. 2009; Duker et al. 2010; Bieth et al. 2015; Fontana et al. 2017). Clinical reports from these cases show that they exhibit all the core features of Prader-Willi syndrome (Table 1.1). All individuals showed severe neonatal hypotonia accompanied by a poor suck reflex, weak cry and a general failure to thrive. Growth was delayed through childhood and left the individuals with short stature in adulthood. Excessive weight gain started at approximately 18-36 months of life, before the development of hyperphagia and all the related behaviours like food foraging and hoarding. Sexual development was delayed, and hypogonadism was present in all cases but the one reported by Butler et al. (1996). Abnormal sleep patterns and sleep apnea were exhibited by all, as well as mild-to-moderate learning disability including poor abstractions skills, deficits in verbal intelligence and short term memory defects. Maladaptive behaviours including aggression, stubbornness, temper tantrums and skin-picking were also reported in all cases. An autism diagnosis was reported only in Sahoo et al. (2008). ADD/ADHD relevant behaviours have not been reported on, with the exception of the Butler et al. (1996) case, which showed no indications of attentional problems.

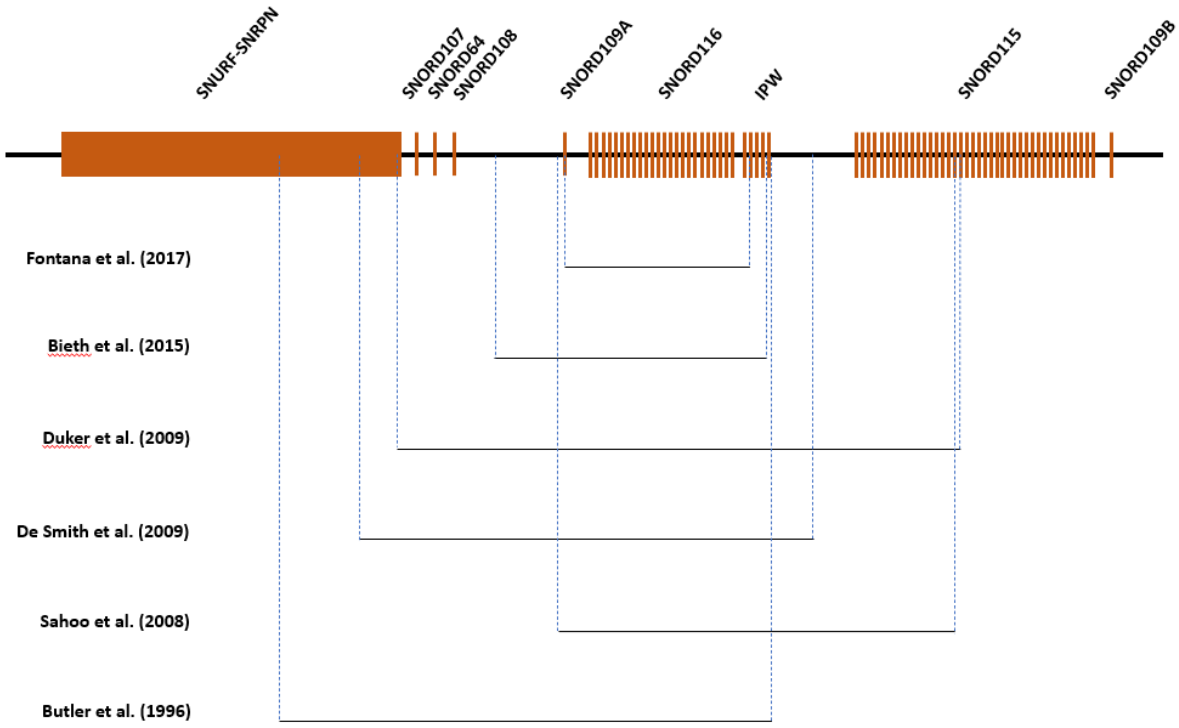


Figure 1.2 Reported cases of microdeletions spanning PWS-cr. In most of these cases, the deletion also includes *SNORD115* or *SNRPN*, both of which are likely to also contribute to psychiatric and behavioural phenotype. Only in the cases reported by Fontana et al. (2017) and Bieth et al. (2015), the patients carry a deletion that includes only *SNORD116, IPW* and *SNORD109A.*

Table 1.1 Phenotypical characterisation of individuals carrying microdeletions that span the PWS-cr interval.

	Hypoton reduced growth	Hyperph obesity	Sexual maturat delay	Disturbed sleep	Cognition	ADD/ ADHD	Maladapt behaviour	Affective disorders	Psychosis
Fontana et al. (2017)	√ & √*	√ & √	√	√	√	-	√	-	X
Bieth et al. (2015)	√ & √	√ & √	√	√	√	-	√	-	X
Duker et al. (2009)	√ & √	√ & √	√	√	√	-	√	-	X
de Smith et al. (2009)	√ & √	√ & √	√	-***	√	-	√	-	X
Sahoo et al. (2008)	√ & √	√ & √	√	√	√	-	√	-	X
Butler et al. (1996)	√ & X**	√ & √	√	√	√	X	√	-	X

* phenotype is present, ** phenotype is absent, *** no reports on the phenotype.

No indications of psychosis were observed in any of these individuals, but it is of note that all cases except for the one reported by Bieth et al. (2015) were under the age of twenty and the cases reported by Butler et al. (1996), Sahoo et al. (2008), and Duker et al. (2009), were under the age of 12 at the time of examination, while the mean age of onset of psychosis for both genetic subtypes is between 21 and 22 years (Soni et al. 2008). There were no reports on the presence of anxiety or depression in any of these individuals.

The molecular role of PWS-cr has been studied with the use of mouse models carrying a deletion of the interval. An RNA-sequencing study of adult cortex tissue lacking PWS-cr showed a dysregulation of over six thousand genes (Powell et al. 2013). Among the genes with affected expression was *Mtor*, which is a key factor in cellular metabolism and skeletal muscle morphology (Bodine et al. 2001; Shimizu et al. 2011). The circadian regulators *Clock, Cry1,* and *Per2* were also differentially expressed in the tissue lacking PWS-cr. A study of the same mouse model shows a loss of diurnal rhythmic DNA methylation in the mouse cortex in the absence of PWS-cr (Coulson et al. 2018). Furthermore, RNA-sequencing studies of adult hypothalamus show over 800 differentially expressed genes between PWS-cr mice and wild type littermates, including enrichments for cellular organisation, development, and growth (Polex-Wolf et al. 2018; Pace et al. 2020b). Overall, studies of the collective role of the critical interval in humans and mouse models show it plays a significant role in the main symptoms of PWS, including hyperphagia, metabolism, obesity, circadian rhythms, cognition and maladaptive behaviours.

1.2.1 Molecular function of SNORD116

SNORD116 (previously known as MBII-85) is a cluster of repetitive snoRNAs, which includes 29 repeats in the human genome (Castle et al. 2010; Good and Kocher 2017). *SNORD116* belongs to a cluster of repetitive snoRNAs, which are all classified as “C/D box snoRNAs”. The C and D boxes are short conserved sequence elements, which allow snoRNAs to regulate site-specific methylation of ribosomal RNA (rRNA) (Sridhar et al. 2008; Makarova and Kramerov 2009; Cavaillé 2017). This process is essential for the maintenance of proper ribosomal function as it regulates the maturity and stability of rRNA. However, none of the C/D box snoRNAs of the PWS region have a direct rRNA target (Makarova and Kramerov 2011), and studies of their functional role have given contradictory results.

One of the hypotheses suggests that the PWS snoRNAs are left untrimmed from long non-coding RNAs (lncRNAs) found between them, thus forming a new class of non-

coding RNAs – sno-lncRNAs. These sno-lncRNAs formations are thought to associate with Fox2 from the Fox family, which is a known regulator of splicing (Yin et al. 2012). Furthermore, one of the other snoRNAs in the PWS region, *SNORD115*, has been suggested to play a regulatory role in the alternative splicing of the 2C serotonin receptor, because its C/D box sequence is complementary to the alternative exon of the transcript (Kishore and Stamm 2006b). This led Kishore and Stamm (2006a) to speculate that snoRNAs play a role in modulating alternative splicing. However, a study of a mouse model carrying a knockout of *Snord115* did not find any evidence of the snoRNA playing such a role in vivo (Hebras et al. 2020), so the role of the PWS snoRNAs in alternative splicing remains contentious.

Crucially, *SNORD116* has been shown to be expressed in NPY neurons in the hypothalamus and in the suprachiasmatic nucleus; areas which are known to play a crucial role in metabolism, hunger and circadian rhythms (Baver et al. 2014; Lassi et al. 2016b; Qi et al. 2016b). Currently, the direct molecular target of *SNORD116* is unknown. Its recognition sites are not complementary to any known rRNA, tRNA or snRNA sequence (Ding et al. 2008). However, *SNORD116* has been demonstrated to have an effect on the expression levels of multiple genes. A study by Falaleeva et al. (2015) on HEK 293T cells and post-mortem hypothalamic cells of PWS patients demonstrated that *SNORD116* affects the expression of over 200 genes. A very recent study on hypothalamic neuronal cell culture showed that *Snord116* regulates the levels of *Nhlh2* by increasing post-transcriptional stability, which suggests a potential molecular mechanism through which the snoRNA might be having an effect on gene expression (Kocher et al. 2021), but this idea requires further substantiation. Although the literature suggests that *SNORD116* has a crucial role regulating gene expression and cellular processes that affect neurodevelopmental outcomes with adverse phenotypical effects, its molecular role is still not understood.

1.2.2 Molecular function of IPW

The other gene in the PWS-cr interval, Imprinted in Prader-Willi (*IPW),* is a lncRNA, which has three exons in the human genome and five exons in the mouse genome. The molecular function of *IPW* is also not entirely clear, although one study suggests that it is involved in the regulation of another cluster of imprinted genes — *DLK1-DIO3*, which is located on chromosome 14 (Stelzer et al. 2014). Stelzer et al. (2014) demonstrated that *IPW* interacts with G9A histone methyltransferase, which catalyses lysine 9 histone H3 tri-methylation (H3K9me3) (Figure 1.3). Pluripotent stem cells from individuals with PWS (PWS-iPSCs) were shown to exhibit significantly

decreased levels of H3K9me3 at a region that maintains the imprinting of the MEGs of the *DLK1-DIO3* locus. Thus, *IPW* maintains repressive chromatin marks on the maternally expressed genes (MEGs) of the *DLK1-DIO3* region. The phenotype was fully rescued by the re-introduction of *IPW* into the PWS-iPSCs which suggests that deletion of *IPW* leads to overexpression of the MEGs in the *DLK1-DIO3* locus.

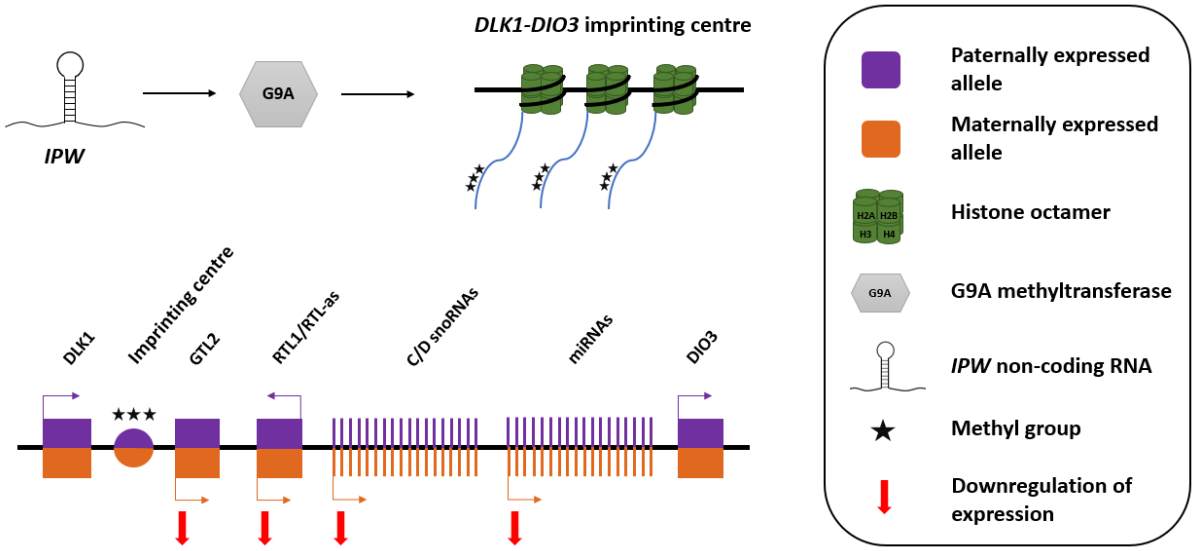


Figure 1.3 *IPW* downregulates the expression of maternally expressed genes in the *DLK1-DIO3* locus. *IPW* downregulates the expression of the MEGs by recruiting G9A histone methyltransferase. G9A adds repressive methyl groups on lysine 9 of the amino acid tail of histone 3 on the chromatin of the imprinting centre of *DLK1-DIO3,* which regulates MEG expression.

Yet another level of interaction between the genes of the PWS-cr interval and the *Dlk1-Dio3* locus was observed in a study of diurnal methylation in a mouse model carrying a deletion of PWS-cr (LaSalle et al, 2018). LaSalle et al. discovered that the maternally expressed genes of the *Dlk1-Dio3* locus lose diurnal methylation marks in the absence of PWS-cr and hypothesized that this process is disrupted by the absence of *Snord116*, although the study by Stelzer et al. (2014) suggests that it is more likely that *Ipw*, rather than *Snord116,* is responsible for that phenomenon.

Notably, the *DLK1-DIO3* imptinted locus has been linked to phenotypes similar to those observed in PWS. Individuals who carry mUPD of chromosome 14, and as a result have an overexpression of the MEGs of *DLK1-DIO3*, exhibit developmental delay, hypotonia, short stature, mild-to-moderate learning disability, precocious puberty and anxiety (Healey et al. 1994; Coviello et al. 1996; Falk et al. 2005). Collectively, these symptoms are clinically recognized as Temple syndrome, although they are often misdiagnosed as PWS in infancy (Kagami et al. 2017). Furthermore,

individual knockouts of the MEGs of the *DLK1-DIO3* cluster in mice have linked the *miR-379/miR-410* miRNA cluster to anxiety and neonatal metabolism, while *Mico1* has been linked to circadian oscillations (Labielle et al. 2008; Labialle et al. 2014; Marty et al. 2016). Overall, this suggests that the *DLK1-DIO3* MEGs might play a key role in the core phenotypical outcomes of PWS.

1.2.3 PWS-cr mouse model

The reported cases of individuals carrying deletions of PWS-cr, as well as studies of the individual genes of the interval, suggest that PWS-cr is the main contributer to PWS phenotypes. A mouse model carrying a deletion of PWS-cr has been extensively studied in order to further our understanding of the role of the interval. It is of note that although the coverage of the deletion is confirmed to span all copies of *Snord116,* it is not clear how much of *Ipw* is deleted. It also has to be taken into consideration that the mouse PWS-cr is distinct to the human PWS-cr: the *Snord116* snoRNA has >40 repeats compared to 29 in human, and *Ipw* has 5 exons instead of 3 (Figure 1.4) (UCSC browser, h38/mm10). Regardless of these differences, the PWS-cr mouse model replicates the core features of PWS.

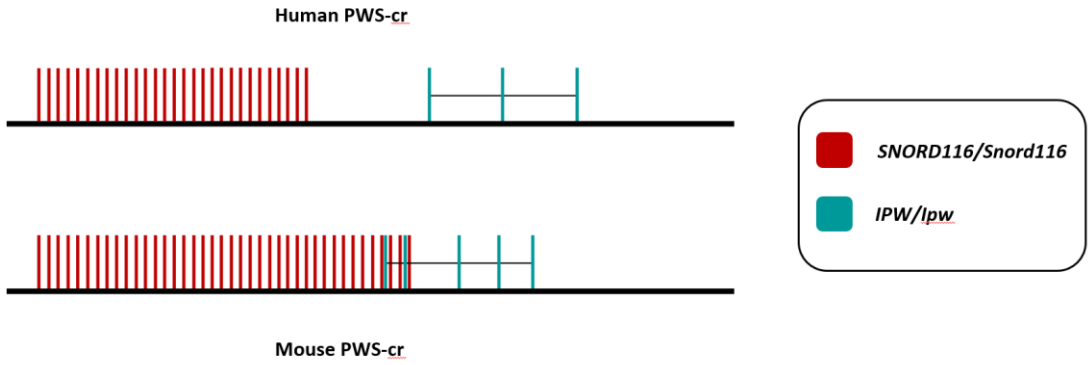


Figure 1.4 Differences between PWS-cr in humans and mice. In the human PWS-cr region,*SNORD116* has 29 copies and the *IPW* gene contains 3 exons. In mice, there are >40 copies of *Snord116*, and *IPW* has 5 exons, two of which overlap with the *Snord116* cluster.

The PWS-cr mice exhibit increased lethality of up to 15%, severe growth retardation starting around P5-P6, and delayed vaginal opening, which is indicative of an overall delay in sexual maturation (Skryabin et al. 2007; Ding et al. 2008; Qi et al. 2016a; Polex-Wolf et al. 2018; Adhikari et al. 2019). Disturbance of circadian rhythms, including decreased daytime activity and increased night time activity and loss of rhythmicity of lipid oxidation, have also been observed (Skryabin et al. 2007; Powell et al. 2013; Qi et al. 2016a), along with alteration of rapid eye movement sleep cycles,

abnormal theta waves and a reduction in ventral hippocampal grey matter, which is crucial for regulating theta rhythms (Lassi et al. 2016b; Pace et al. 2020a; Pace et al. 2020b).

Reports of the role of PWS-cr for hyperphagia in mice have been contradictory. A few studies have shown that mice carrying a germline deletion of the critical interval exhibit increased food intake and failure to reduce consumption when introduced to a high fat diet (Ding et al. 2008; Lin et al. 2014; Qi et al. 2016a). These behavioural changes are accompanied by structural changes in the lateral hypothalamus, elevated levels of circulating ghrelin hormone, neuropeptide Y (NPY) and POMC, as well as dysregulation of orexin neurons (Skryabin et al. 2007; Qi et al. 2016a; Pace et al. 2020b), which have a role in food intake and metabolism (Barson et al. 2013; Burdakov et al. 2013). In contradiction of the evidence above, two studies have reported no indications of hyperphagic behaviour in the PWS-cr mouse model (Powell et al. 2013; Polex-Wolf et al. 2018).

PWS-cr mice also have a significantly reduced fat content and increased lean mass on a regular and on a high fat diet (Ding et al. 2008; Powell et al. 2013; Qi et al. 2016a). They show an increased energy usage, which accounts for some of the increased feeding and reduced fat content (Skryabin et al. 2007; Powell et al. 2013; Qi et al. 2016a).

Interestingly, the differences in the NPY and POMC system were corrected when mice were kept at an increased temperature of 30°C (Qi et al. 2017). Furthermore, the PWS-cr mice kept at ambient temperatures had reduced energy expenditure and increased body weight gain. The differences in food consumption previously observed in the same lab (Qi et al. 2016) were also reversed. Crucially, deletion of PWS-cr in adults leads to opposite phenotypical outcomes to the ones observed in the germline deletion model. Adult onset deletion of PWS-cr induced increased adiposity, followed by *hypo*phagia (Purtell et al. 2017). However, PWS-cr deletion specific to the adult mouse hypothalamus, on the other hand, resulted in both hyperphagia and obesity with increased adiposity (Polex-Wolf et al. 2018).

Overall, increased feeding in PWS-cr is associated with lean body composition and vice versa. The reduction of fat storage in mice requires an increase in energy expenditure for thermogenesis. This suggests that the observed hyperphagic behaviour might be a compensatory feature for alterations in body composition, rather than a replication of the satiation phenotype, which is typical for individuals with PWS.

While the body composition and feeding behaviour of the PWS-cr mouse model have been extensively studied, behaviours relevant to cognition and psychiatric illness have been underexplored. There is currently only one published study on the cognition of this mouse model, which investigated response to novel objects and reported impairment in novel object recognition and object location memory (Adhikari et al. 2019). Furthermore, Adhikari et al. reported a delayed development of neurological reflexes such as the righting reflex after being placed in supine position. In terms of behaviours relevant to psychiatric illness, only anxiety has been investigated at the elevated plus maze and open field tasks, which has shown some indications of elevated anxiety (Skryabin et al. 2007; Zieba et al. 2015; Adhikari et al. 2019).

1.2.4 PWS-IC mouse model

Behavioural phenotypes of relevance to psychiatric illness and cognition have been demonstrated in a 'full' mouse model of PWS carrying a deletion of the imprinting center (PWS-IC) (Relkovic et al. 2010). This deletion leads to loss of imprinting and, in turn, overexpression of the MEGs and loss of expression of all the PEGs (PWS-cr included) which replicates the expression profile of the mUPD15 genetic subtype. Relkovic et al. (2010) showed that PWS-IC mice exhibit no indications of anxiety as examined by the open field test, but have an increased acoustic startle response, impaired pre-pulse inhibition and deficits in attention, as studied by the 5-choice serial reaction time task (5-CSRTT) (Relkovic et al. 2010).The impulsivity of the PWS-IC model has also been tested through the stop-signal reaction time task, in which the mice exhibited deficits in response inhibition, which are indicative of impulsive behaviour that's commonly shown by individuals with PWS (Davies et al. 2019).

1.4 Aims

The main aim of this work is to contribute to the understanding of the molecular biology behind the cognitive and psychiatric phenotypes of Prader-Willi syndrome. The focus of this study is to determine whether the critical interval, in particular, plays a role in shaping the cognitive and psychiatric profile of individuals with PWS. One of the ways in which this is examined is by repeating the behavioural experiments previously done on the PWS-IC model with the PWS-cr mouse model, and where appropriate, drawing comparisons between the two since the former also exhibits loss of expression of PWS-cr among other genes. By repeating the behaviours previously studied in the PWS-IC mouse model in the PWS-cr mouse model, I aim to ellucidate

whether the loss of expression of the critical interval contributes to the phenotypes observed in the PWS-IC model.

Since the behavioural study identified a distinct phenotypic profile of the PWS-cr mice compared to that of the PWS-IC mice, the next aim was to examine the gene expression profiles that underlie these distinct behavioural phenotypes in order to disentangle the genetic mechanisms behind them. Furthermore, the loss of expression of PWS-cr has been shown to be the main cause of the core features of PWS (Sahoo et al. 2008; de Smith et al. 2009; Duker et al. 2010; Bieth et al. 2015; Hassan and Butler 2016; Fontana et al. 2017). Therefore, discovering its molecular targets could be instrumental for the development of therapies that aleviate the deabilitating symptoms of this disorder.

Finally, since the results from behavioural and gene expression studies indicated that PWS-cr might have a role in intellectual disability and depression, I aimed to research this further with the use of human genomic data.

The specific aims of this thesis were as follows:

1) **To study the behaviour of the PWS-cr mouse model** in aspects of stress and anxiety (Chapter 2), psychiatric illness (Chapter 2), and attention and impulsivity (Chapter 3), and to determine whether the absence of the critical interval contributes to behavioural endophenotypes of relevance to psychiatric illness and cognition, which have been previously observed in the PWS-IC mouse model.
2) **To perform a transcriptomic study on whole brain neonatal samples from both PWS-cr and PWS-IC mouse models** in order to investigate whether the critical interval has a regulatory role on gene expression relevant to cognition and psychiatric illness (Chapter 4), and to observe how the PWS-cr neonatal brain transcriptomic profile compares to the transcriptomic profile of the PWS-IC mouse.
3) **To examine whether variation within the critical interval is linked to phenotypes relevant to cognition and psychiatric illness in humans** through a study of UK biobank data (Chapter 5).

Chapter 2. Characterisation of basic behaviours of the PWS-cr mouse model

2.1 Introduction

Prader-Willi Syndrome (PWS) is associated with a distinctive behavioural profile, including temper tantrums, aggression, obsessive-compulsive tendencies, and skin-picking, which are cumulatively linked to a range of affective and psychotic disorders (Cassidy et al. 2011; Angulo et al. 2015). Individuals with PWS exhibit a highly increased prevalence of anxiety disorders compared to the general population, and over 70% of them score highly on behavioural checklists for anxiety (Dykens and Shah 2003; Soni et al. 2007; Skokauskas et al. 2012). Psychotic episodes are also reported in upward of 19% of individuals (Vogels et al. 2004; Soni et al. 2007; Thuilleaux et al. 2018a).

Family and twin studies demonstrate heritability of generalised anxiety disorder (Mendlewicz et al. 1993; Scherrer et al. 2000; Hettema et al. 2001), with the serotonin transporter being implicated as one of the candidate genes (You et al. 2005; Chang et al. 2017). Notably, a previous study demonstrated reduced functionality of the 2C serotonin receptor in the PWS-IC mouse model. Furthermore, two molecular studies of the same mouse model propose that the paternally expressed *SNORD115* snoRNA from the PWS locus regulates alternative splicing mechanisms and A-to-I editing of the 2C serotonin receptor gene (Doe et al. 2009; Garfield et al. 2016). This suggests that *Snord115* might play a role in elevated anxiety observed in individuals with PWS. Since *Snord116* from the PWS critical interval has been shown to regulate the expression of *Snord115* (Falaleeva et al. 2015), it is possible that PWS-cr also plays a role in phenotypes of anxiety.

Individuals with PWS also exhibit psychotic behaviour including paranoid ideation, persecutory delusions and/or hallucinations. Similar to anxiety, psychotic behaviours have been shown to be heritable and rooted in genetic and epigenetic causes, with *CACNA1A* and *ZN804A* being considered two of the main genes responsible, (O'Donovan et al. 2009; Zhao et al. 2015). A number of recent genomic studies linked variants in the PEGs from the PWS locus *MAGEL2, NECDIN* and *SNORD116*, to scoring highly on the Schizotypal Personality Questionnaire (Crespi et al. 2018; Salminen et al. 2020), which suggests a potential role for the PWS PEGs, and, crucially to this study, for PWS-cr in psychiatric illness.

Since studies have demonstrated that the PWS-IC mouse model recapitulates behavioural phenotypes of relevance to the psychiatric illness (Relkovic et al. 2010), I have repeated the behavioural tests from the PWS-IC mouse studies on a mouse

model carrying a deletion only of the PWS-cr interval, to investigate whether PWS-cr contributes to the behaviours exhibited by the PWS-IC mouse model.

The elevated plus maze (EPM) and open field (OF) tests were used to examine anxious behaviour. The EPM and OF tests explore the natural inclination of rodents to avoid open spaces (File 2001; Carola et al. 2002). Less time spent in the open and anxiogenic areas of the OF and EPM are associated with higher levels of anxiety (File 2001). Locomotor activity was also studied within and separately from the OF and EPM arenas in order to discern the potential confounding effects of hypotonia and hypoactivity on the movement of the mice within the two mazes.

The acoustic startle response (ASR) and pre-pulse inhibition (PPI) of the acoustic startle response were used to test for endophenotypes of relevance to psychiatric illness. The startle response is a bodily muscle convulsion elicited by an intense sensory stimulus. This phenomenon is thought to be an adaptation developed in mammals as a part of the fight-or-flight response and is increased in the presence of threats and fear (Davis et al. 1993; Koch 1999; Hebb et al. 2003). Deficiencies in the startle response and failure to habituate to startling stimuli have been reported in a range of different psychiatric and affective disorders (Shalev et al. 1992; Akdag et al. 2003; Ludewig et al. 2005). When a stimulus of high intensity is preceded by exposure to a stimulus of lower intensity (pre-pulse), the startle response is stunted. The pre-pulse allows the nervous system to adapt to the stimulus of higher intensity. This phenomenon is known as sensory-motor gating. Defects in pre-pulse inhibition are also found in different psychotic and affective disorders (Parwani et al. 2000; Hoenig et al. 2005).

Overall, the aim of this chapter is to investigate the role of the PWS-cr interval for behaviours relevant to the psychiatric illnesses observed in individuals with PWS.

2.2 Methods

2.2.1 Animal generation and husbandry

Mice carrying a paternal PWS-cr deletion were purchased from The Jackson Laboratory (B6.Cg-*Snord116*$^{tm1.1Uta}$/J), which generated the animals from the Ding et al. (2008) model. The mice were delivered in breeding pairs along with wild type males and females of the same B57BL/6 background. Female PWS-cr mice were crossed with male wild types in order to generate heterozygous PWS-cr mice that carry the deletion on the maternal line. Since PWS-cr is only paternally expressed, heterozygous mice carrying the deletion on their maternally inherited copy of chromosome 15 have normal PWS-cr expression. After that, the behavioural cohort was generated by breeding the heterozygous males carrying a PWS-cr deletion on the maternal lineage with wild type CD1 females in order to replicate the PWS-IC behavioural cohort, which was generated by crossing CD1 wild type females and B57BL/6 PWS-IC males in order to reduce the unsustainable lethality rate. The wild type littermates were used as a control group. Both breeding rounds produced a twofold number of females compared to males, which encumbered the generation of a sufficient number of males for testing sex-genotype interactions.

Weaning took place at approximately four weeks of age, when the animals were housed in single sex and mixed genotype cages of 2-4. Experimental testing began at approximately 8-9 weeks of age. The animals were handled daily for two weeks prior to that in order to acclimatize them to being picked up. Vaginal swabs were taken on the day of each behavioural test in order to account for the potential effect of oestrus phases on behaviour. Animals were weighed weekly from P21 onwards.

2.2.2 Genotyping

At approximately 28 days of age, tissue of 2 mm diameter was taken from the ears of the mice for genotyping and identification. The tissue was dissolved overnight at 55˚C in 300 µl of tail lysis solution (100 mM Tris HCl pH 8.5, 5 mM EDTA pH 8, 0.2% SDS, 200 mM NaCl, 10 µg/ml proteinase K), then vortexed and centrifuged at 14 000 rpm for 10 min to remove pelleted debris. 300 µl of ice-cold isopropanol were added to the supernatant and the solution was left at -20˚C for an hour, before centrifugation at 14 000 rpm for 5 min to collect a pellet. 300 µl 70% ethanol were added to the pellet and centrifuged at 14 000 rpm for 5 min. The resulting pellet was dried off of ethanol at 50˚C and dissolved in 50 µl of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0).

For PCR amplification, 1 µl of DNA in TE was added to 20 µl of reaction mix (Table 2.1) before the PCR was run (94°C for 10 min, 35 cycles of [94°C for 20 s, 60°C for

20 s, 72°C for 90 s], followed by 72°C for 5 min). The amplified DNA segments (435 bp - wild type, 337 bp – PWS-cr) were run through 1.5% agarose gel at 100V for 1 h.

Table 2.1 PCR reaction mix.

Reaction component	Concentration
Primer 1 AAT CCC CAA CCT ACT TCA AAC AGT C	0.5 µM
Primer 2 TGG ATC TCT CCT TGC TTG TTT TCT C	0.5 µM
Primer 3 TTT ACG GTA CAT GAC AGC ACT CAA G	0.5 µM
dNTPs	0.25 µM
PCR buffer (Qiagen) 5x	1x
HotstarTaq (Qiagen) (5U/µl)	1 U/µl
ddH_2O	Up to final volume

2.2.3 Elevated plus maze

The EPM consists of a plus shaped platform, with two enclosed arms (19x8x15 cm) and two open, exposed arms (19x8 cm) (Figure 2.1a). The maze is lifted 50 cm above the ground and illuminated at 15 lux. The animals were gently placed in one of the enclosed arms of the maze at the beginning of the task, and allowed to freely explore the terrain for 5 min. The movement of the animals in the maze was detected by a camera (17 frames/s) mounted above the maze and linked to a computer running an Ethovision XT software (Noldus, NL), which virtually divides the EPM into 5 zones; 2 closed arms, 2 open arms and a centre square at the cross section of the arms. The software tracked three points on the body of the mice; front, middle and centre. Mice were recorded as being inside an area when the middle point was in that area. Other behaviours such as grooming and risk assessing as exhibited by head-dips over the edge of the open arm and stretch-attends forward into the open arms were recorded manually. The main measures analysed at the EPM as indicative of anxiety levels were percentage time spent in the open arms of the maze (out of total time spent in the arms of the EPM – time in the centre was excluded), distance travelled, and number and duration of occasions of grooming, head dipping and stretch attending.

2.2.4 Open field test

The OF test consists of a square arena (75x75 cm) enclosed by walls (45 cm) and illuminated from above at 15 lux. The surface of the arena is divided into central zone (45x45 cm) and outer zone (Figure 2.1b). The animals were placed into the outer zone of the arena and allowed to freely explore the terrain for 10 min. The movement of each subject inside maze was detected by Ethovision XT software with the same

camera and body tracking settings as the EPM. The main measures analysed at the OF test as indicative of anxiety were percentage of time spent in the centre (out of total time) of the field and total distance travelled.

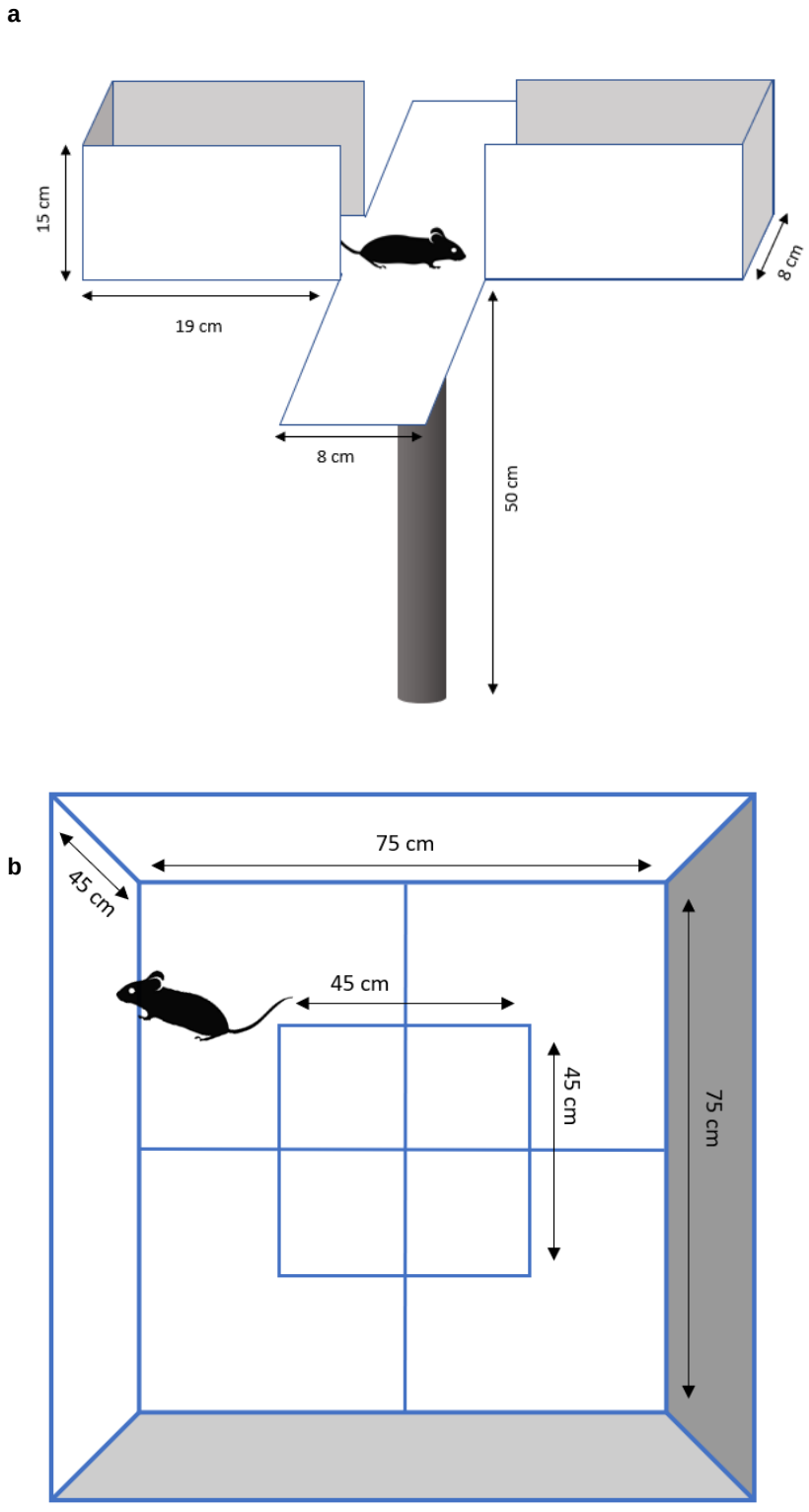


Figure 2.1 Setup for the elevated plus maze (a) and open field test (b). The dimensions of the two apparats.

2.2.5 Locomotor activity test

Spontaneous locomotor activity (LMA) of mice was measured in chambers (21x36x20 cm) fitted with infrared beams, which are situated 3 cm from either end of the box and 1 cm from the floor of the box (Figure 2.2a). The test was run in the dark in order to remove the anxiogenic effect of light. The mice were placed in the boxes and allowed to roam freely for 2 h, while the disturbance of the beams by their movement was recorded by an Arachnid software (Cambridge Cognition LTD). The disturbance of the two separate infrared beams consecutively (referred to as a "run") was treated by this study as the main measure of locomotor activity. The animals were assessed at the same time for two consecutive days, in order to assess habituation to a novel environment within and between sessions.

2.2.6 Acoustic startle response and pre-pulse inhibition

The apparatus for measuring ASR and PPI consists of a plexiglass tube (3.5 cm in diameter) contained in a soundproofed SR LAB startle chamber (San Diego Instruments) (Figure 2.2b). The animals were placed securely in the tube and were then allowed to habituate to the environment for 5 min with background white noise of 70 dB, generated by a speaker inside the chamber. After habituation, the animals were exposed to acoustic stimuli, including startle stimuli alone at 120 dB and 105 dB for 40 ms, and startle stimuli preceded by "pre-pulses" of 8 dB and 16 dB for 20 ms, 100 ms prior to startle stimuli (Figure 2.1c). All startle and pre-pulse stimuli were presented against a background noise of 70 dB. The startle response in response to acoustic stimuli was recorded by a piezoelectric pressure-sensitive accelerometer. All startle responses were normalized by body weight. PPI of the ASR was calculated as a percentage reduction in startle response when the startle stimuli were pre-empted by a pre-pulse versus when the startle stimuli were presented alone.

2.2.7 Statistical analysis

All data were analysed using R Studio 1.1.383 (R Studio, Inc) by linear models (lms) and generalised linear models (glm) using genotype and sex as fixed effect. Data calculated in percentages were analysed using logistic glms from the binomial family. The oestrus cycle phase variable was tested separately on female subjects with lms and glms. Since no effect on behaviour was observed, the variable was not added to the overall statistical models. Repeated measures tests were analysed with mixed lms and glms with mouse ID added as random effect, in the ASR, PPI, and LMA tests, with the use of the lme4 package (Bates et al. 2015).

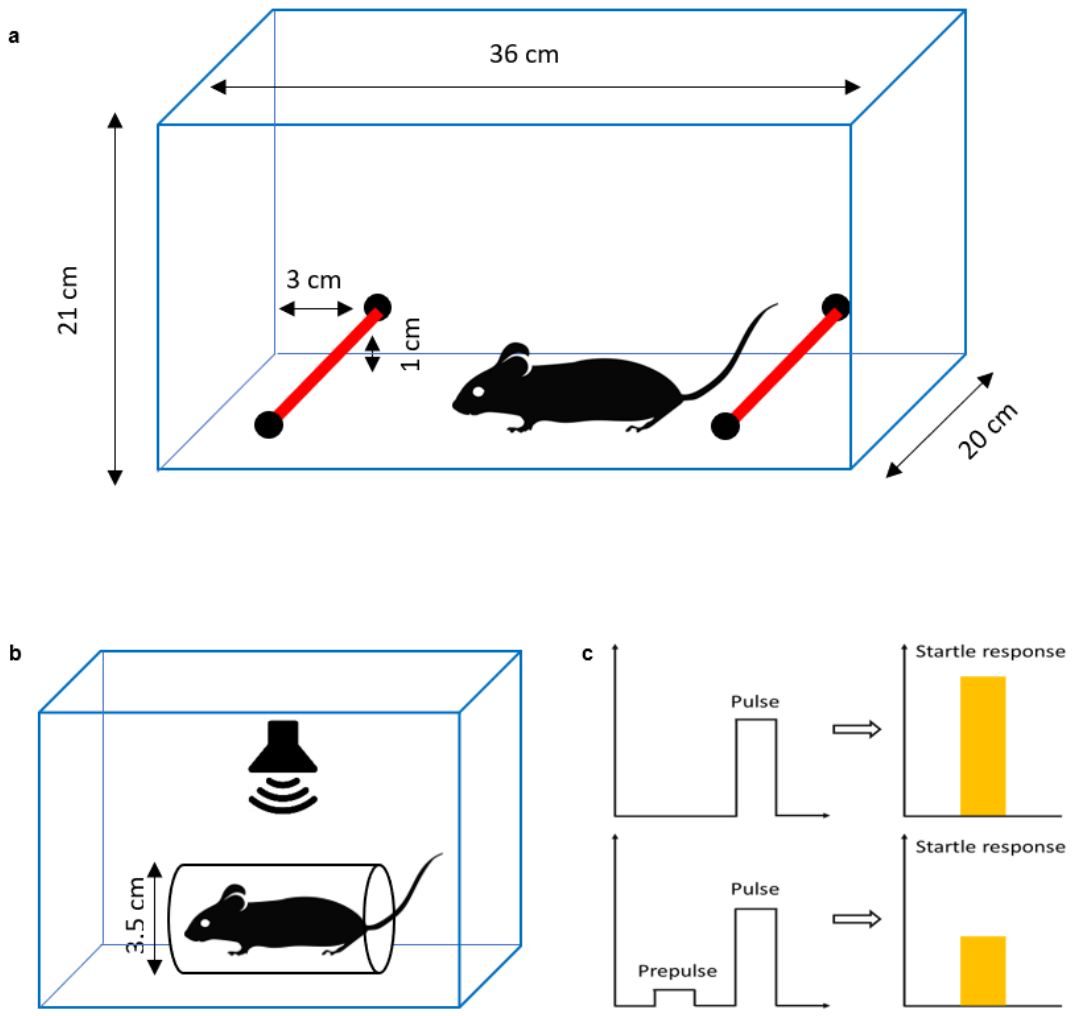


Figure 2.2 Locomotor activity test, acoustic startle and pre-pulse inhibition tests. The LMA test was conducted in a dark box fitted with two infra-red beams that tracked the movement of the mice (a). The ASR and PPI tests were conducted in a dark sound-proof box, where pulses of different intensity were played to induce a startle response (b). Pre-pulses of lower intensity were played prior to some of the pulses to check the pre-pulse inhibition response (c).

The test results presented for 'repeated measures'/mixed models were calculated by using the anova function in R, using chi square to compare the full statistical models to reduced models with the variable of interest removed, as permitted by Wilks' theorem (Wilks 1938). For the LMA test, day of testing and time bins were also analysed as within-subject factors. For ASR, the startle trial number was analysed as within-subject factor. For PPI, gating at different pre-pulse decibels (8 dB, 16 dB) were analysed as within subject factors. Since there was no observed effect of sex on genotype in any of the analysed parameters with the exception of body weight, the effect of sex was only reported in that section.

2.3 Results

2.3.1 Body weight from juvenility to adulthood

Although the main goal of this chapter is to investigate behaviour, I also analysed the growth of this mouse from juvenility to adulthood since the PWS-cr has been considered responsible for the hypotonia and hyperphagia observed in individuals with PWS. In order to investigate physical growth and body weight gain, mice were weighed weekly for eight weeks from the point of weaning (week 3 to week 11 post birth). The results showed that as expected females had a significantly lower weight than males ($X^2_{(2)}$ = 63429, p<0.001), and PWS-cr mice had significantly lower weight than wild type mice ($X^2_{(2)}$ = 7.451, p=0.024) (Figure 2.3). Surprisingly, there was a significant interaction between genotype and sex ($X^2_{(1)}$ = 3.941, p=0.047) since the effect of genotype on growth was present only in the females and not in the males. This finding has not been previously reported in studies of the PWS-cr mouse model.

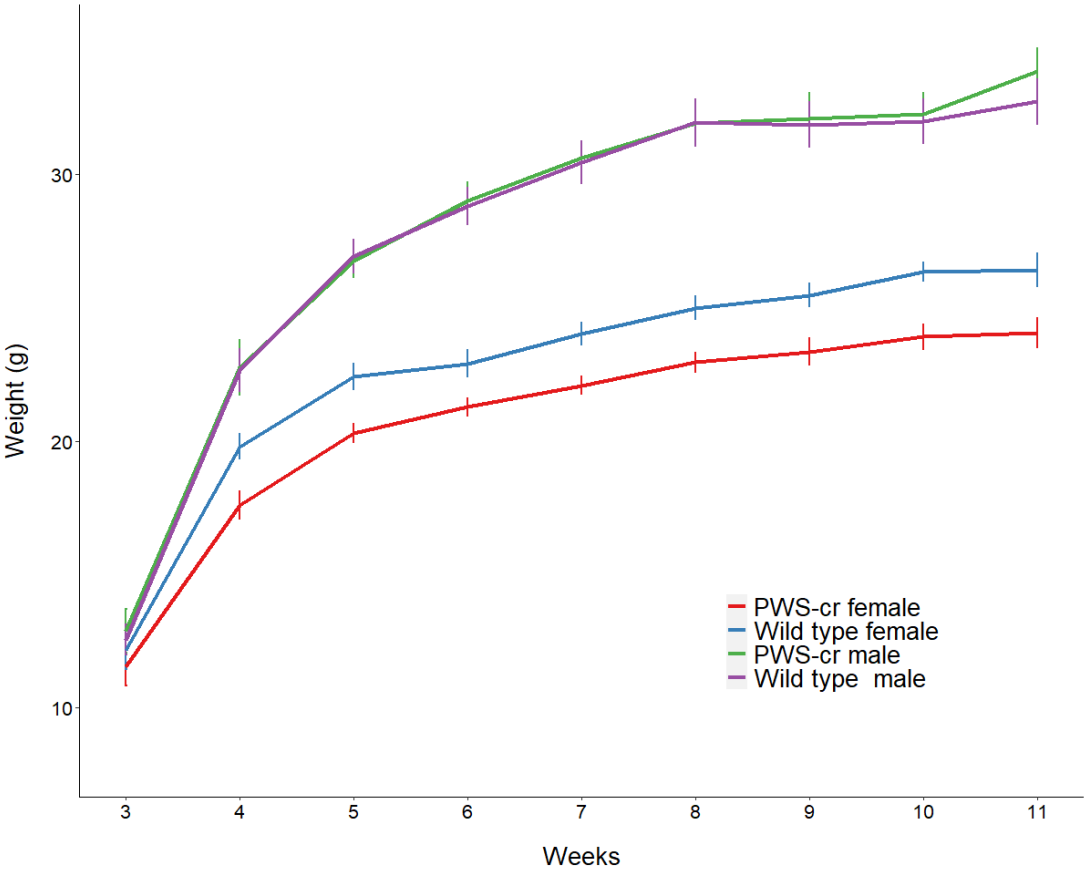


Figure 2.3 Body weight from juvenility to adulthood. The body weight of mice from 3 to 11 weeks post birth was significantly affected by both sex (p<0.001) and genotype (p=0.024). Female PWS-cr mice (n=14) maintained significantly lower weight than female wild types (n=17), while the weight of male PWS-cr mice (n=7) was not significantly different to that of male wild types (n=14). Error bars represent SEM.

2.3.2 Elevated plus maze and open field behaviours

The EPM and OF tests were used to investigate anxiety-related phenotypes in 5 min and 10 min sessions, respectively. As expected, in the EPM all mice spent significantly more time in the closed arms of the maze compared to the more anxiogenic open arms of the maze ($X_{(1)}$=138.98, p<0.001), but there was no effect of genotype on percentage of time spent in the open arms of the EPM ($F_{(3,50)}$=0.132; p=0.361), or on distance travelled through the area of the maze ($F_{(3,\ 50)}$=2.352, p=0.174) (Figure 2.4a, 2.4b). Number and duration of head dips, stretch attends and grooming occasions were also recorded as a measure of explorative behaviour, and showed no significant differences between PWS-cr mice and their wild type littermates (Head dip: $F_{(3,\ 50)}$=0.7832, p=0.989; $F_{(3,\ 50)}$=0.553, p=0.300; Stretch attend: $F_{(3,\ 50)}$=1.933, p=0.151; $F_{(3,\ 50)}$=1.917, p=0.237; Grooming: $F_{(3,\ 50)}$=0.146, p=0.651; $F_{(3,\ 50)}$=0.3, p=0.41) (Figure 2.4c, 2.4d).

In the OF test, mice spent significantly more time in the outer areas of the arena than in the more anxiogenic centre square ($X_{(1)}$=452.2, p<0.001), but there was no effect of genotype on percentage of time spent in the centre square or on distance travelled in the arena ($F_{(3,\ 50)}$=0.084, p=0.361; $F_{(3,\ 50)}$=4.55, p=0.472) (Figure 2.4e, 2.4f). Overall, the data collected from the EPM and OF show no differences in behaviour between PWS-cr mice and their wild type littermates and indication of anxiety phenotype in the PWS-cr mouse model.

2.3.3 Locomotor activity

LMA was measured in custom made chambers fitted with infrared beams tracking the movement of the mice. The test was run in a two-hour session for two consecutive days. There was no effect of genotype on total number of beam breaks made over the span of the two sessions ($X^2_{(2)}$ =4.625, p=0.099) (Figure 2.5a), or on total number of runs made from one side of the chamber to the other ($X^2_{(2)}$ =1.237, p=0.539) (Figure 2.5c). Further analysis of the data in 30 min time bins showed that all animals exhibited habituation to the environment within and between sessions, as indicated by the steady decrease of number of beam breaks ($X^2_{(3)}$ =197.83, p<0.001; $X^2_{(1)}$ =33.562, p<0.001) (Figure 2.5b) and number of runs over time ($X^2_{(3)}$ =347.75, p<0.001; $X_{(1)}$=49.953, p<0.001) (Figure 2.5d). However, there was no significant difference in habituation between the two genotypes ($X^2_{(2)}$ =3.342, p=0.342; $X^2_{(2)}$ =1.761, p=0.222). Overall, the results from this test showed no evidence of a locomotor activity phenotype in the PWS-cr mice.

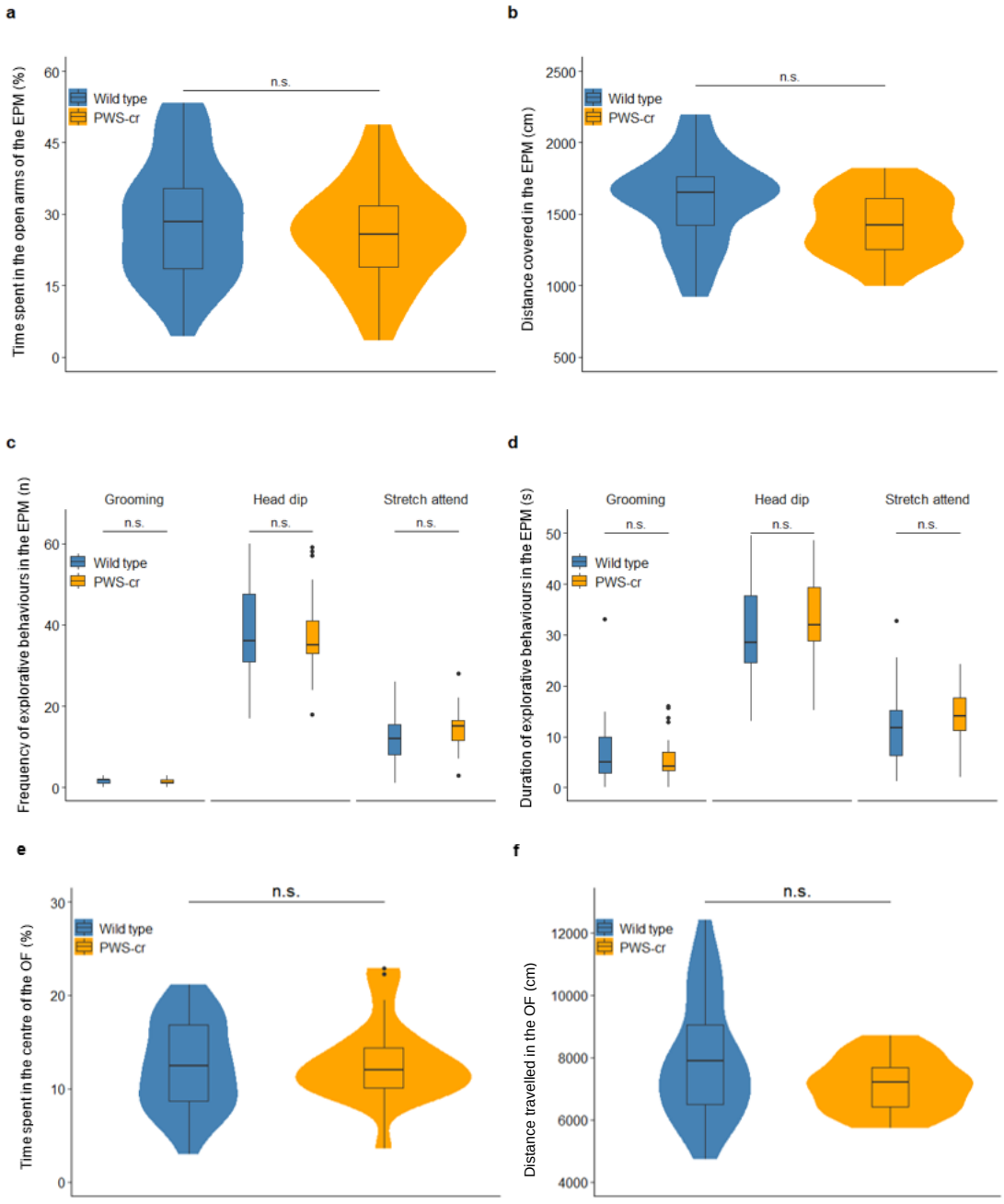


Figure 2.4 Results from the elevated plus maze and open field test. Results from the EPM showed no significant difference between PWS-cr mice (n=23) and wild type mice (n=31) on percentage of time spent in the open arm of the maze (p=0.361), or distance travelled through the maze (p=0.174) (a, b). No effect of genotype was found on number and duration of head dips, stretch attends and grooming occasions (c, d). Similarly, data from the open field test showed no difference in percentage of time spent in the centre square of the arena or total distance covered throughout the session (e, f).

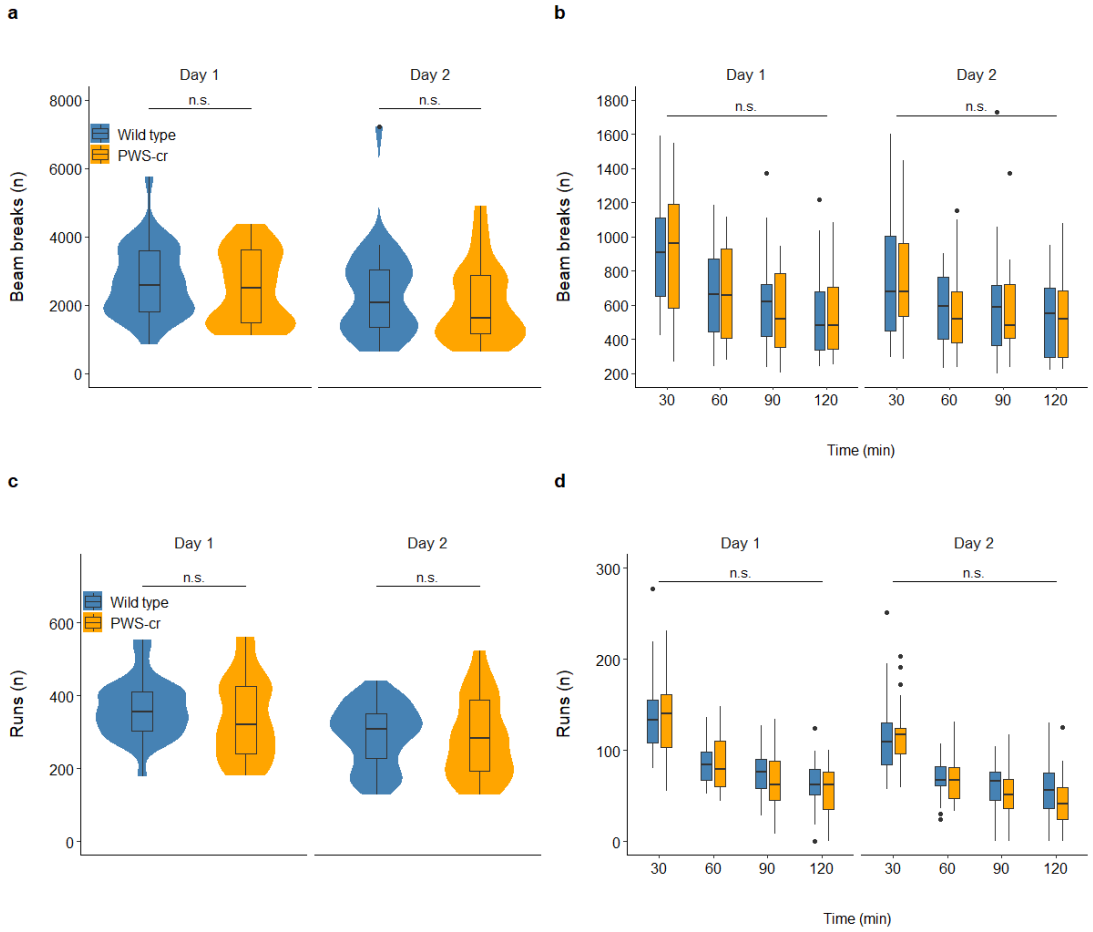


Figure 2.5 Results from the locomotor activity test. Data from the LMA test showed no significant difference between the total number of beam breaks and runs performed by PWS-cr mice (n=23) and wild type mice (n=31) across two consecutive days (p=0.099, p=0.539) (a, c). A breakdown of the beam break and run data into 30 min bins indicated within session habituation to the environment (p<0.001, p<0.001), which was not affected by genotype (p=0.426) (b, d). All significance indicatives on the figure show differences between the genotypes.

2.3.4 Acoustic startle and pre-pulse inhibition

ASR and PPI were measured at pulse intensities of 120 dB and 105 dB. A total of thirteen ASR-alone pulses were presented to the mice at the two intensities, and both showed a significantly reduced startle response in the PWS-cr mice ($X^2_{(2)}$=6.5292, p=0.03821; $X^2_{(2)}$=14.562, p<0.001) (Figure 2.6a, 2.6b). A look at the startle responses at 10 dB increment increases of stimuli from 70 dB to 120 dB shows a positive correlation between startle response and noise intensity in both genotypes ($X^2_{(12)}$ =253.28, p<0.001) (Figure 2.6e). Furthermore, the difference between PWS-cr and wild type only appeared at noise intensity stronger than 100 dB and both groups were equally sensitive to noises of lower decibels, which suggests that the reduced startle response observed in PWS-cr mice is likely not due to impaired hearing.

Pre-pulses of 8 dB and 16 dB inhibited the startle response by mean average of 52.46% (±3.14% SEM) and 69.87% (±3.48% SEM) for pulses of 120 dB, and 39.28% (±5.53% SEM) and 50.69% (±5.61% SEM) for pulses of 105 dB. This indicates operational sensory-motor gating in the cohort (Figure 2.6c, 2.6d). Ultimately, genotype had no effect on pre-pulse inhibition at either pulse intensity ($X^2_{(2)}$=5.376, p=0.251; $X^2_{(2)}$=2.556, p=0.635). Overall, the results from the ASR and PPI tests show no sensory-motor gating phenotypes in the PWS-cr mouse model, but a reduced startle response to acoustic stimuli.

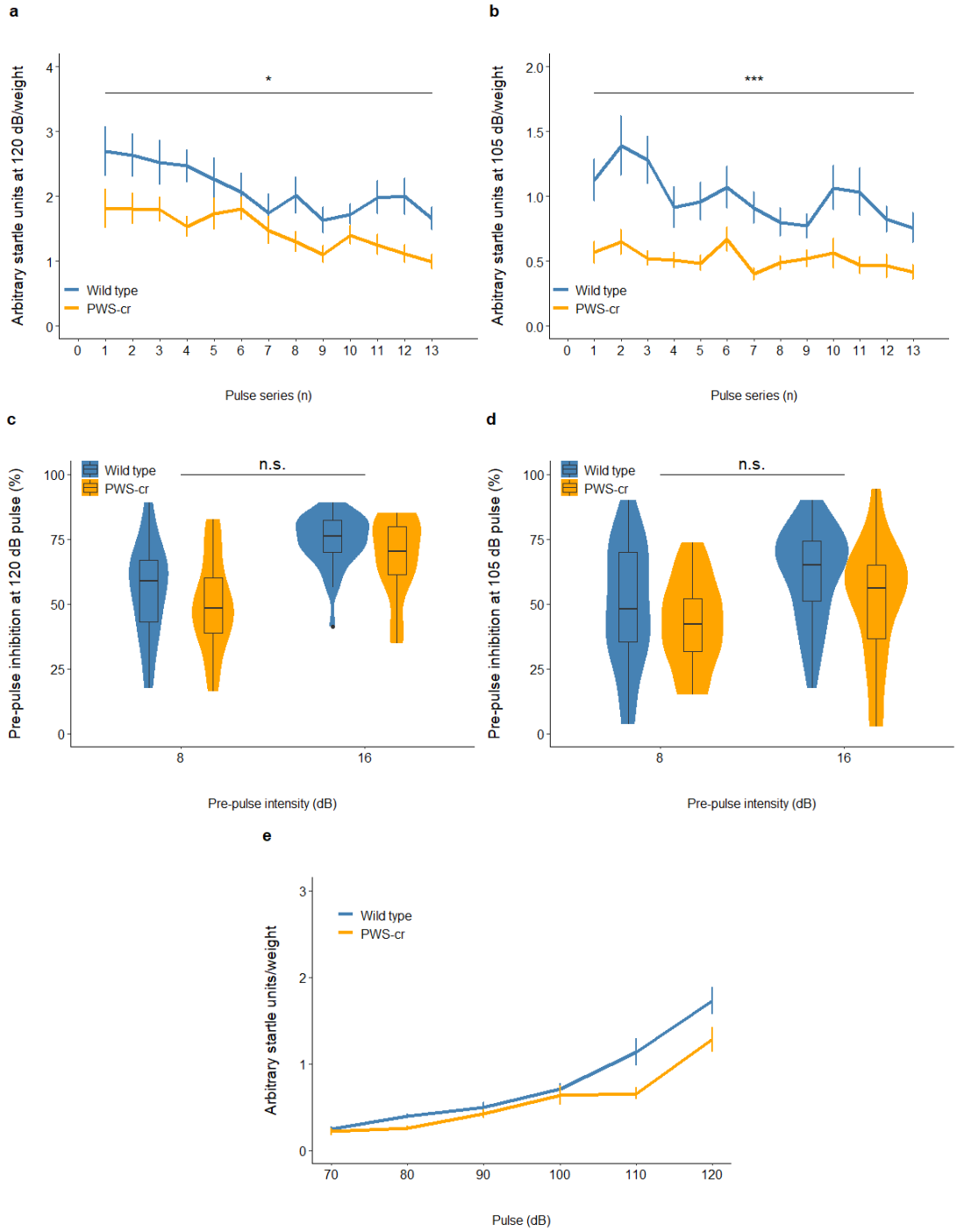


Figure 2.6 Results from the acoustic startle response and pre-pulse inhibition tests. The ASR to a series of 13 stimuli at 120 dB (a) and 105 dB (b) was significantly reduced in PWS-cr mice (n=23) compared to their wild type littermates (n=31). PPI of the ASR was measured by presenting stimuli of 8 dB and 16 dB 100 ms before the stimuli of 120 dB (c) and 105 (d). The pre-pulse inhibition data showed operational sensory motor gating, which was not affected by genotype. Startle responses at 10 dB increment increases of stimuli from 70 dB to 120 dB shows a positive correlation between pulse intensity and startle response, but no difference between genotypes at pulse intensity equal to or lower than 100 dB. * indicates $p<0.05$, *** indicates $p<0.001$, error bars indicate SEM. All significance indicatives on the figure show differences between genotypes.

2.4 Discussion

This chapter describes the behavioural phenotypes and growth of the PWS-cr mice. The examination of this model suggests that the deletion of the critical region has a sex-dependent effect on growth. In terms of behaviour, the PWS-cr mice exhibited no indicatives of anxiety as measured by the EPM and OF tests, no alterations to locomotor activity, and no sensory-motor gating phenotypes as measured by PPI. Only the results from the ASR test showed a significantly reduced startle response to acoustic stimuli in the PWS-cr mice compared to wild type littermates, which has been associated with a range of psychiatric disorders (Shalev et al. 1992; Akdag et al. 2003; Kaviani et al. 2004; Hoenig et al. 2005).

2.4.1 Impaired growth in the PWS-cr mice

The growth of the PWS-cr mouse model has already been thoroughly documented in several studies (Ding et al. 2008; Lassi et al. 2016a; Qi et al. 2016a). As expected, the PWS-cr mice used for this study consistently exhibited lower body weight than wild type from juvenility to adulthood (3 weeks to 11 weeks post birth). This suggests that the PWS-cr interval might be contributing to the reduced growth phenotype observed in PWS-IC mice (Relkovic et al. 2010), and in individuals with PWS (Cassidy et al. 2011; Angulo et al. 2015). This hypothesis is further supported by a few reported cases of individuals who carry a deletion spanning the PWS-cr and exhibit hypotonia and low weight in infancy (Butler et al. 1996; Sahoo et al. 2008; Duker et al. 2010; Bieth et al. 2015; Fontana et al. 2017). A candidate gene for the impaired growth phenotype is *IPW* — a known regulator of a cluster of micro RNAs in the *DLK1-DIO3* locus on chromosome 14 (Stelzer et al. 2014), which have been associated with Temple syndrome and consequently hypotonia and reduced growth in the first 2-3 years of life (Kagami et al. 2017).

Notably, the impaired growth phenotype was only observed in the females and not in the males. This finding has not been previously reported in studies of the PWS-cr and PWS-IC mouse models, nor has it been observed in individuals with PWS. One possible explanation for this disparity is that it's a feature of the strain cross generated by breeding B57BL/6 heterozygous males and wild type CD1 females, which, to our knowledge, has not been used in other studies of this mouse model. It also has to be acknowledged that the male group had the smallest n due to an overall low number of males in the offspring of this mouse model compared to females. It is therefore possible that the interaction between sex and genotype is a type I error caused by low statistical power for examining sex-genotype interactions. However, this

interaction between sex and genotype was not observed in any of the other behaviours studied in this chapter.

2.4.2 PWS-cr mice exhibited no indications of anxiety in the elevated plus maze and open field test

This study showed no significant differences between PWS-cr mice and their wild type littermates in any of the examined aspects of behaviour in the OF and EPM. Previous studies of this mouse model have used the OF and EPM to study anxiety phenotypes with conflicting results. Zieba et al. (2015) discovered behaviours indicative of reduced anxiety in PWS-cr mice in the OF test, and of increased anxiety in the EPM, whereas Ding et al. (2008) found no effect of the deletion in the OF and increased anxiety in the EPM.

This disparity between the results of this chapter and the literature could be due to differences in testing conditions, to which both EPM and OF are notoriously sensitive, particularly in regard to light settings (Lewejohann et al. 2006; Sousa et al. 2006). It is of note that the PWS-IC mouse model that was previously examined in the same conditions in our lab and was shown to carry distinctive behavioural phenotypes and deficiencies in all of the other tests, also showed no significant differences in behaviour in the OF (Relkovic et al. 2010). A possible explanation is that settings used in the study presented here are excessively anxiogenic, and thus incite the bottom-line threshold of behaviour under the conditions of the test, leaving no room for differences between the genotypes. However, the set up in our lab has previously yielded differences in both directions compared to studies of the same models in literature (Westacott 2017), which suggests that insensitivity due to the apparatus is unlikely. It is worth mentioning, that the main parameter for behaviour in the EPM (%time spent in the open arms of the maze), excludes time spent in the centre of the maze from the analysis, which could potentially be concealing aspects of anxious behaviour. In future studies, it might be worth trying the elevated zero maze instead, since it does not have this problem and might be a better paradigm for studying anxiety (Singh et al. 2007).

2.4.3 No evidence of a locomotor activity phenotype in the PWS-cr mice

The locomotor activity of PWS-cr mice and wild type littermates was examined over 2 h session, in two consecutive days and no effect of genotype on locomotor activity was observed. Patterns of habituation of mice to the LMA boxes were also examined and analysed. The absence of the PWS-cr interval had no effect on habituation to the novel environment. Comparatively, the PWS-IC mice exhibited both significantly

reduced locomotor activity and deficient habituation to the LMA boxes (Relkovic et al. 2010), which reflect hypoactivity phenotypes in individuals with PWS (Cassidy et al. 2011; Angulo et al. 2015).

2.4.4 Reduced acoustic startle response in the PWS-cr mice, but no effect on pre-pulse inhibition

The ASR of PWS-cr mice was significantly lower than that of their wild type littermates. Comparatively, the PWS-IC mice, who also exhibit a loss of PWS-cr expression, had a significantly higher startle response than their wild type littermates (Relkovic et al. 2010). Since PWS-IC mice have a disrupted expression of over ten genes, including the two genes contained in the PWS-cr interval, these contrasting outcomes of the startle response test are not too surprising considering the complexity of the neural circuitry behind these phenotypes. Ultimately, both increased and reduced ASR have been reported in a range of psychiatric and affective disorders such as depression, post-traumatic stress disorder, eating disorders, anxiety and psychosis (Shalev et al. 1992; Akdag et al. 2003; Kaviani et al. 2004; Ludewig et al. 2005).

The PPI showed no significant difference between PWS-cr and wild type mice. In comparison, the PWS-IC mouse model has been shown to have reduced PPI (Relkovic et al. 2010), which demonstrates impaired sensory-motor gating and has been associated with psychotic disorders, mainly schizophrenia (Parwani et al. 2000; Powell et al. 2009; Ziermans et al. 2011). The results from this study of the PWS-cr mouse suggest that the reduced PPI observed in PWS-IC mouse is likely not caused by the loss of expression of the PWS-cr interval.

Overall, the combined phenotype of an increased startle response and deficient pre-pulse inhibition seen in the PWS-IC mouse model is linked to psychotic illness (Parwani et al. 2000; Akdag et al. 2003). Consequently, the lack of any PPI deficit in PWS-cr deletion model suggests that their reduced startle response is a distinct behavioural outcome, although it is also possible that the reduced startle response in the PWS-cr mice masks any sensory-motor gating impairments, since the startle is low to begin with and there is no much room for significant reduction when the pre-pulse is introduced. This could be further tested in future studies by modifying the ASR settings to increase the power of the stimulus in order to try and increase the startle response in the PWS-cr mice.

Whilst increased ASR has been linked to stress, pain and fear (Sorenson and Swerdlow 1982; Davis et al. 1993; Hebb et al. 2003), reduced startle response has

been reported in individuals with major depressive disorder and anhedonia, as well as an effect of anxiolytic drugs (Riba et al. 2001; Commissaris et al. 2004; Kaviani et al. 2004). With this in mind, the reduced startle endophenotype seen in PWS-cr mice could fit the psychiatric profile of individuals with the delPWS genetic subtype, who are prone to depression and anxiety, but rarely psychosis (Soni et al. 2008; Sinnema et al. 2011). However, further behavioural analysis is required to properly establish a depression phenotype in the PWS-cr mice.

It should be acknowledged, that reduced acoustic startle results could potentially be confounded by hearing loss. However, a look at the startle response curves at increasing pulse intensity does not indicate hearing loss. There are no records of a hearing loss phenotype in individuals with PWS, or in any mouse models of PWS. Furthermore, testing took place when the mice had reached around 9-10 weeks of age, so age-related hearing loss is also unlikely.

2.4.5 Conclusion

In conclusion, the behavioural studies conducted in this chapter showed subtle effects of PWS-cr deletion on behaviour. The acoustic startle response was the only affected behaviour, and while it's possible that the PWS-cr interval plays a role in depressive phenotypes, it does not seem to be responsible for the full behavioural profile of the PWS-IC mouse model, or the corresponding psychotic illness commonly observed in individuals with PWS.

Chapter 3. Characterisation of behaviours at the 5-choice serial-reaction time-task

3.1 Introduction

Populational studies of individuals with PWS report phenotypes of reduced attention, hyperactivity and impulsivity, similar to the symptoms of attention-deficit/hyperactivity disorder (ADHD) (Gross-Tsur et al. 2001; Wigren and Hansen 2005; Reddy and Pfeiffer 2007). While the behaviours related to appetite and psychiatric illness have been extensively characterized, research into the comorbidity of PWS with ADHD is sparse.

A meta-review of over a hundred studies including 171,756 subjects estimated that the prevalence of ADHD among children and adolescents worldwide is approximately 5% (Polanczyk et al. 2007), whereas in individuals with PWS the percentage goes up to 25% (Gross-Tsur et al. 2001; Wigren and Hansen 2005). It is of note that the average individual with PWS scores between 60 and 70 on IQ tests (Yang et al. 2013), thus often covering the criteria of a lower than 70 point threshold for an intellectual disability (ID) diagnosis (Vissers et al. 2016), which in itself has a comorbidity with ADHD of 8%-15% (Dekker and Koot 2003; Emerson and Hatton 2007). However, when directly compared, individuals with PWS exhibit ADHD-like symptoms at significantly higher rates than their peers diagnosed with ID only (Reddy and Pfeiffer 2007).

ADHD is a highly heritable disorder, with children and siblings of affected individuals having an up to tenfold increased risk of developing the disorder (Biederman et al. 1990; Biederman et al. 1992). Furthermore, twin studies suggest that between 70% and 80% of the phenotypes associated with ADHD are due to genetic variability, as opposed to environmental factors (Larsson et al. 2014; Faraone et al. 2015). In fact, a meta-analysis of twin and adoption reports indicated that out of several psychopathologies including depression, anxiety and disruptive behaviour, ADHD was the only one to come out as largely uninfluenced by environmental factors (Burt 2009).

Genome-wide association studies (GWAS) of genomic variants have struggled to identify loci with single nucleotide polymorphisms (SNPs) robustly associated with ADHD. Recently, a GWAS study pinpointed 12 loci that were correlated with the disorder, but the small odds ratios of association suggest that these loci only reveal a small percent of the genetic risk factors for ADHD (Demontis et al. 2019). Copy number variations (CNVs) have also been associated with the disorder, and a couple

of those CNVs in particular fall in the 15q11.2-15q13 interval, within the PWS locus (Williams et al. 2010), which suggests a genetic, and not just phenotypic overlap between PWS and ADHD.

Despite of the prevalence of ADHD in individuals with PWS, the genetics underlying the disorder have not been explored. Phenotypes of attention and impulsivity have been investigated only in the PWS-IC mouse model (Relkovic et al. 2010). An extensive study of the PWS-IC mouse model's visuospatial attention and response control has previously been performed using the five-choice serial reaction time task (5-CSRTT) (Relkovic et al. 2010). The results of the study indicated that the PWS-IC model recapitulates phenotypes of inattention, which are a part of the PWS clinical profile. However, it is still unclear which of the dysregulated genes in the PWS-IC mouse model play a role in the development of these behaviours.

The aim of the study presented in this chapter is to examine the collective effect of the *Snord116* and *Ipw* genes on attention and impulsivity through the use of the 5-CSRTT. The choice serial reaction paradigm was first utilized to test human attentional processes (Wilkinson 1963), but has since been developed for use on mice and rats to investigate models of ADHD (Humby et al. 1999; Bari et al. 2008), as well as of substance abuse and binge eating, (Belin et al. 2008; Voon et al. 2014). The 5-CSRTT is performed in operant boxes with five response locations, where stimulus control is tested after discrimination training. Subjects are trained to respond to visual stimuli arising within the response locations for a food reinforcement. Throughout the training process, the stimuli duration is reduced from session to session, until "baseline" parameters are reached, at which point the conditions of the task are considered appropriate to test behaviours relating to attention and impulsivity (Robbins 2002; Bari et al. 2008). After performance at "baseline" conditions is assessed, the task can be manipulated to further tease out aspects of attention and impulsivity by modifying the length of the stimulus duration and the intervals between trials. Thus, the 5-CSRTT has become an invaluable tool in studying rodent models of ADHD.

The experiment reported in this chapter investigates phenotypes relating to attention and impulsivity of a mouse model carrying a deletion of the PWS-cr interval. These behaviours are examined by recording various elements of behaviour at the 5-CSRTT, including accuracy of responses to stimuli, number of premature responses, reaction time and correct response latency. The study aims to shed some light on the molecular and genetic basis of ADHD-like symptoms in individuals with PWS.

3.2 Methods

3.2.1 Restricted water access regime

A total of 50 animals were used for the 5-choice serial-reaction time task: 23 carrying a deletion of the PWS cr, and 27 wild type litter mates. At 10-11 weeks of age the animals were put on a restricted home cage water access regime in preparation for the 5-CSRTT, in order to increase interest in the condensed milk dilution provided as a reinforcement in the task, and to provide motivation to work for the reward. Limited access to water was introduced two weeks before commencing any experimental tasks in order to ensure adjustment to the restriction. During the first two days of this regime, animals were given access to water for 4 hours per day. After that, the access to water was limited to 2 hours per day. Water bottles were weighed before and after access hours, in order to confirm consumption. After the first two weeks of adaptation to the regime, behavioural tasks were commenced, at which time water was provided in the home-cages for 2 hours immediately after testing. The weight of the animals was monitored daily throughout the entirety of the water deprivation period, in order to ensure that none of the animals had weighed in on less than 80% of their initial body weight as per Home Office licence regulations.

3.2.2 Reward preference test

Prior to the 5-CSRTT and after successful transition to a restricted home cage water access, animals were gradually exposed to a condensed milk dilution (10% condensed milk in tap water) in order to habituate them to the substance which was to be used as the reward for the tasks later on. The animals were placed individually in a cage without sawdust for a 10 min session each day, over a 7 day period. Each cage contained two small plastic containers (~1 cm high x ~2 cm diameter) secured to the floor by Velcro, placed equidistant from the end wall, and evenly spaced across the width of the cage.

On the first two days, both containers contained tap water, and on the following four days one container contained condensed milk dilution and the other — water, with the positions alternating on each day of testing. On the final day, both containers were filled with the condensed milk dilution. All consumption was measured in grams by weighing the containers before and after the task.

3.2.3 5-choice serial-reaction time-task

The 5-CSRTT was used to measure visuo-spatial attention and impulsivity. The task was carried out using a bank of 9-hole chambers (Figure 3.1, Campden Instruments,

UK), each enclosed in a sound attenuating chamber. Each of the equidistantly spaced holes across the array could present a visual stimulus and also register a nose-poke response by the breaking of an infrared beam. For this task, 4 of the 9-holes in the array (number 2, 4, 6 and 8) were blocked by metal shutters. On the far side of the nose-poke array was a food magazine, to which the reward was delivered by a peristaltic pump, via silicone tubing. Entrances to the reward magazine were also recorded by the breaking of an infrared beam. The chambers could be illuminated by lights in their side walls (house lights), although all sessions were conducted in the dark apart from time out periods (Figure 3.2).

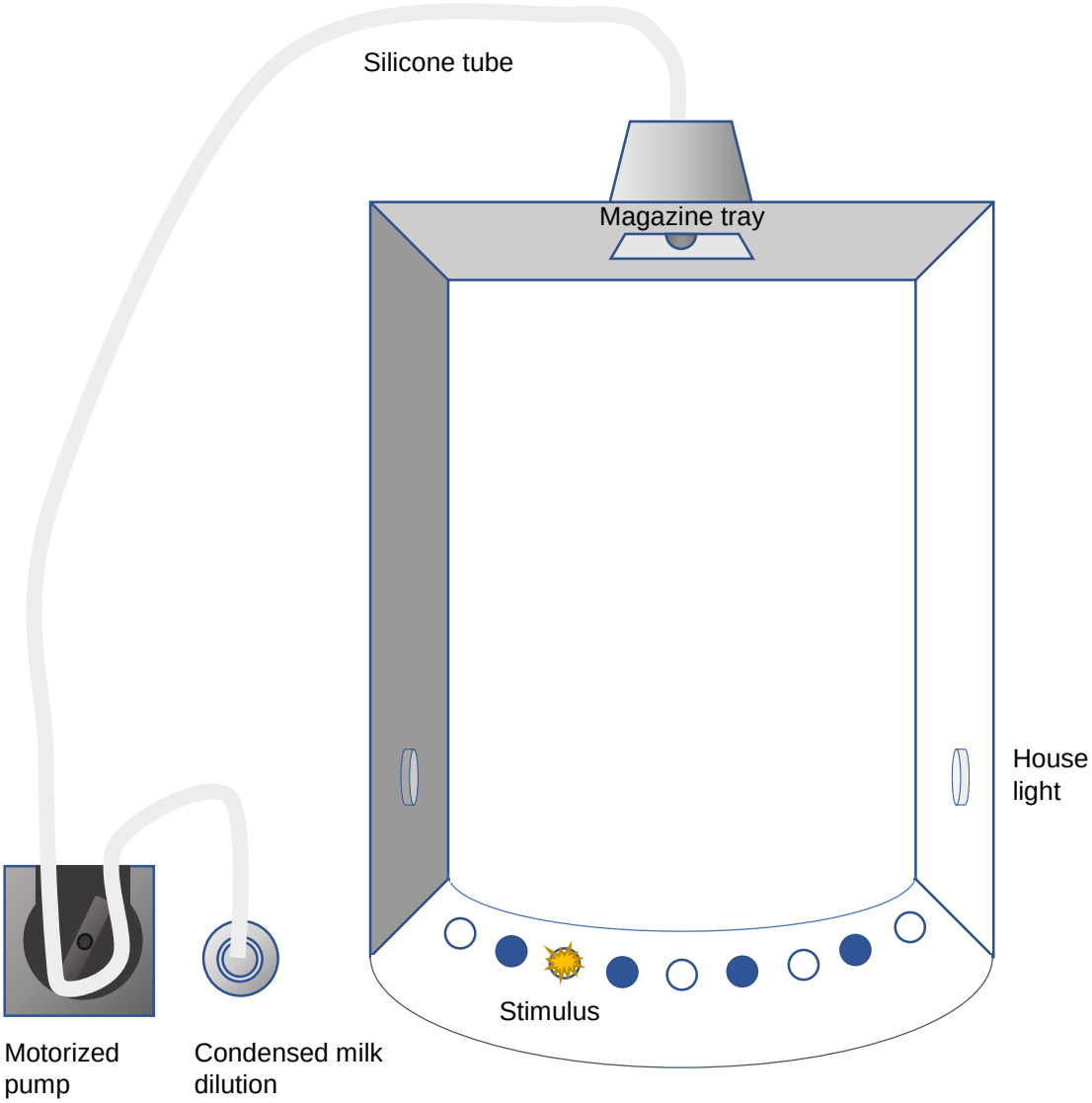


Figure 3.1 9-hole operating box chamber. In the 5-choice serial reaction time task, mice were trained to respond to visual stimuli presented by LED lights inside five of the holes of a 9-hole chamber. A correct response was rewarded by 70 µl of condensed milk dilution, pumped out by a motorized pump through a silicone tube, and presented in a magazine tray inside the chamber.

Replicating previous studies (Humby et al., 2005), the mice were given a single 5-CSRTT session each day, where a session lasted for either a maximum of 60 trials or 20 minutes. Each trial consisted of a stimulus presentation, pseudorandomly presented to one of the 5 locations, a 5 s limited-hold period during which the stimulus was extinguished but the subject could still make a response, and a 5 s inter-trial interval (ITI). A correct response (i.e. a nose-poke at the whole where the stimulus was presented) was rewarded by a 70 µl delivery of the reward, whereas an incorrect response or an omission (i.e. no response) was followed by a 5 s time out period accompanied by illumination of the chamber by the house light. Responding during the ITI was defined as a “premature response”, which initiated a time out and restarted the current trial (Figure 3.2).

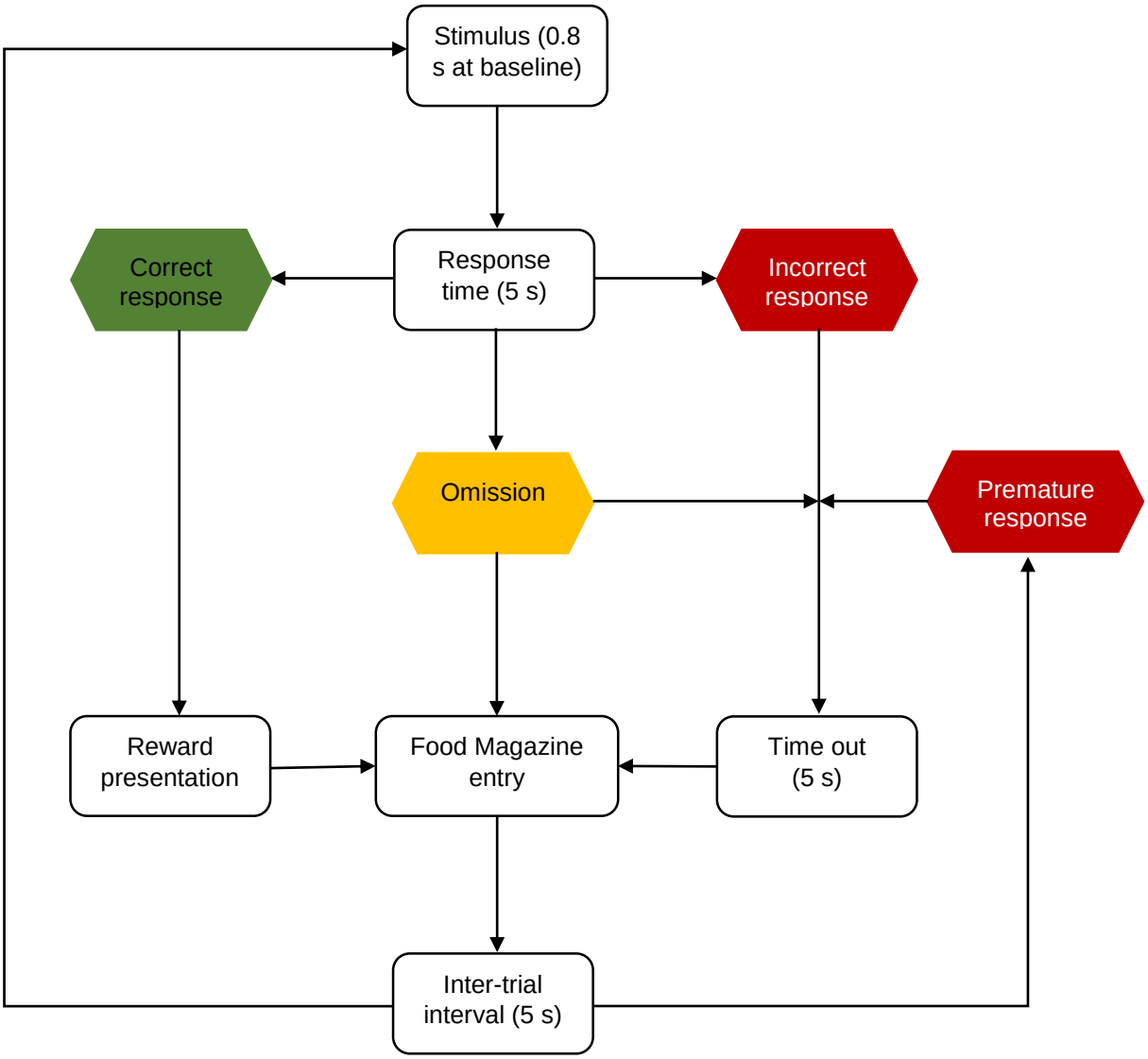


Figure 3.2 5-choice serial-reaction time-task design at baseline conditions. Responses at the 5-CSRTT were divided into four categories (hexagons). A nose poke at the most recently illuminated hole was considered a correct response (highlighted in green) and was rewarded with condensed milk dilution. A nose poke at the incorrect hole or a premature response before the stimulus has appeared (highlighted in red) triggered a switch on of the chamber lights for 5 s. Lack of response within 5 s of the disappearance of the stimulus was counted as an omission (highlighted in yellow). All of the responses had to be followed by an entry of the magazine tray in order to trigger the next stimulus after a 5 s inter-trial interval.

The variables analysed as indicative of performance were accuracy (calculated as percentage correctly performed trials out of total completed trials), omissions (calculated as percentage of omitted trials out of total number of trials performed),

and premature responses (calculated as percentage of premature responses out of total number of trials performed). Completed trials (calculated as percentage out of 60), correct response latency (calculated in seconds), reward collection latency (calculated in seconds), number of food magazine entries and total number of trial perseveration were also recorded for further analysis.

3.2.4 Training and shaping of the 5-choice serial-reaction time-task

The 5-CSRTT was commenced on the day following the final day of the reward preference test and followed the protocol described by (Humby et al. 2005). In the first stage of shaping, the mice were habituated to the chambers for half an hour, during which the reward was presented to the reward magazine every 30 s. Mice completed a minimum of 3 sessions. If they made >35 reward magazine entries in at least two consecutive sessions they were moved on to the next stage of training. Mice remained at this stage until they met the criteria.

In the next stage of training, the mice performed a simplified version of the 5-CSRTT whereby only the central of the five holes was illuminated, with a stimulus duration of 60 s (1-choice serial-reaction time-task). Each session lasted up to 30 minutes or until the completion of 60 trials, which lead to termination of the program. Once a mouse had completed three sessions, it was moved on to the next task if their performance had met criteria (>30 completed trials, >75% accuracy, <25% trial omissions) for two consecutive days. The following task was the 5-CSRTT, with an initial stimulus duration of 60 s. If after three sessions a mouse had met the criteria above for a minimum of two consecutive sessions, the stimulus duration was decreased in the following pattern: 32 s, 16 s, 8 s, 4 s, 2 s, 1,8 s, 1.6 s, 1.4 s, 1.2 s, 1 s and 0.8 s. A stimulus duration of 0.8 s was considered baseline, and once reached, mice were kept on it for a minimum of five sessions and had to meet performance criteria for a minimum of two consecutive sessions, thus showing “stable” baseline performance before task manipulations were implemented.

3.2.5 Task manipulations

To further assess different aspects of attention, impulsivity, and motivation, the mice were presented with a series of manipulations of task parameters (stimulus duration and ITI length) or individual subject state. These manipulations were always presented in the same order and between each manipulation, the mice were placed back on baseline conditions for a minimum of three sessions and had to maintain performance criteria (>30 trials, >75% accuracy, <25% trial omissions) for a minimum of two consecutive sessions before the next manipulation was implemented.

In two of the manipulations, the stimulus duration was increased or decreased to make the task easier or harder, respectively. In the long stimulus duration task (LSD), stimuli durations of 0.8, 1.2, 1.6 and 2 s were presented in pseudorandom order through the session, whereas in the short stimulus duration task (SSD), stimuli of 0.2, 0.4, 0.6 and 0.8 s were presented in pseudorandom order throughout the session. In order to investigate impulsive behaviour, the duration of the ITI was increased in each trial with durations of 5, 7, 9 or 11 s, presented pseudorandomly throughout the session. To investigate the motivational aspect of the task, the mice were given access to water in one session and to the reward in another session, immediately before being tested in the 5-CSRTT at baseline conditions. Mice were housed individually for 10 min immediately before testing, in a small box which contained a measured amount of water or the reward in a small container (~1 cm x ~2 cm) placed at the rear end of an empty cage. Volume consumed was determined by comparing the weight of the container before and after the test.

3.2.6 Statistical analysis

All data were analysed using R Studio 1.1.383 (R Studio, Inc). Quantity of consumption was normalised by metabolic body weight (bodyweight^0.75) (Kleiber 1932; Brown et al. 2004) prior to analysis. Consumption of the reward on day 7 of the test was analysed by a linear models (lm) with genotype and sex as fixed effects. Consumption of water, which was spread over two days was analysed with a mixed lm using the lme4 package (Bates et al. 2015), adding mouse ID as a random effect. The preference to the reward over water was calculated as a percentage of total consumption for days 3 to 6, when the mice were exposed to both simultaneously. The analysis of reward preference was performed using a mixed logistic generalized linear model (glms) from the binomial family with genotype and sex as fixed effects and mouse ID as a random effect.

5-CSRTT data was analysed with lms or logistic glms from the binomial family when the dependant variable was calculated in percentages, using genotype and sex as fixed effects. For analysis of number of training sessions required until baseline conditions were reached, the final session at baseline conditions before the first task manipulation was used. Average number of sessions taken to reach stable baseline between manipulations was calculated by dividing the number of sessions each mouse took to get back to stable baseline by the number of manipulations it completed. The effect of the different manipulations on the number of sessions taken to reach stable baseline was analysed in an lm with mouse ID added as a random

effect. Performance at baseline conditions was calculated by averaging the data from two sessions immediately prior to the first task manipulations and analysed in a lm. In the ITI and stimulus duration manipulations, mouse ID was added as a fixed effect to the lms and glms.

For the analysis of performance at 5-CSRTT baseline conditions after exposure to water or the reward, consumption of water or the reward was added to the lms and glms as a fixed effect. All consumption was measured and normalized by metabolic body weight (body weight^$^{0.75}$). Effect on overall performance after water or reward consumption was analysed in separate lms and glms in comparison to the baseline session that took place on the previous day to the respective manipulation. The test results presented for 'repeated measures'/mixed models were calculated by using the anova function in R, using chi square to compare the full statistical models to reduced models with the variable of interest removed, as permitted by Wilks' theorem (Wilks 1938). Since there was no observed effect of sex on behaviour with the exception of progress through task manipulations, the effect of sex was only reported in that section.

3.3 Results

3.3.1 Reward preference test

In the first two days of the reward preference test, in which the mice were exposed to water only, there was no significant difference in consumption between PWS-cr mice and their wild type littermates (Figure 3.3A; $X^2_{(2)}=2.743$, p=0.254). Consumption on the seventh and final day of the test, in which the mice were exposed to the reward only, also showed no significant difference between genotypes (Figure 3.3B; $F_{(3, 44)}=0.738$, p=0.789). In the four days in which the animals were exposed to both water and the reward, both groups showed preference to reward over time ($X^2_{(3)}=51.935$, p<0.001), thus demonstrating the condensed milk dilution is an appropriate substance for motivational reward. There was no significant effect of genotype on reward preference (Figure 3.3C; $X^2_{(2)}=0.583$, p=0.747).

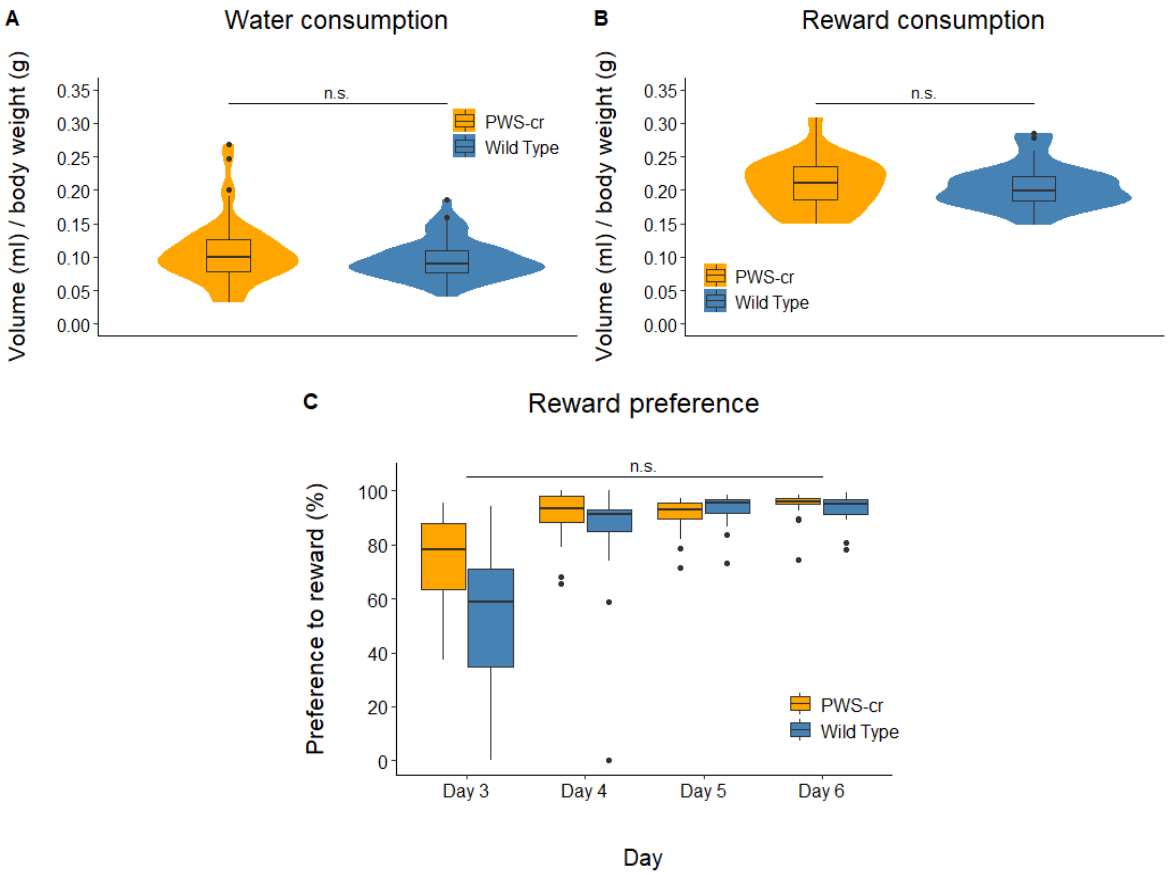


Figure 3.3 Reward preference test. The results from this test showed no difference between PWS-cr mice and their wild type littermates in overall water (A) and condensed milk reward (B) consumption (p=0.254, p=0.789). Preference to reward over water (C) increased over the course of four days in which the PWS-cr (n=23) and wild type mice (n=27) were provided with both (p<0.001), and this was not influenced by genotype (p=0.747). All significance indicatives on the figure show differences between genotypes.

Overall, the data from the reward preference test demonstrated no difference in consumption of the reward between PWS-cr mice and their wild type littermates after normalising for metabolic body weight. These results show no evidence of hyperphagic behaviour or increased interest by the PWS-cr mice toward the reward.

3.3.2 Performance at baseline conditions at the 5-CSRTT

Of the 23 PWS-cr and 27 wild type mice which were allocated to the study, one PWS-cr died of causes unrelated to the experiment at the beginning of the 5-CSRTT training. Furthermore, 2 PWS-cr and 3 wild type mice did not meet the set criteria during training to move on to baseline conditions after a total of 250 sessions and were excluded from the analysis. Performance at baseline conditions showed no significant differences in accuracy, omissions, number of completed trials or number of premature responses (Figure 3.4A-D; $F_{(3,\ 42)}$=0.127, p=0.724, $F_{(3,\ 42)}$=0.104; p=0.749, $F_{(3,\ 42)}$=0.009; p=0.925, $F_{(3,\ 40)}$=0.678, p=0.385). Other parameters such as reward collection latency, number of magazine entries and number of trial perseveration also showed no significant differences between PWS-cr mice and wild type littermates (Figure 3.5B-D; $F_{(3,\ 40)}$=3.643; p=0.125; $F_{(3,\ 40)}$=1.916; p=0.653; $F_{(3,\ 40)}$=1.164, p=0.125).

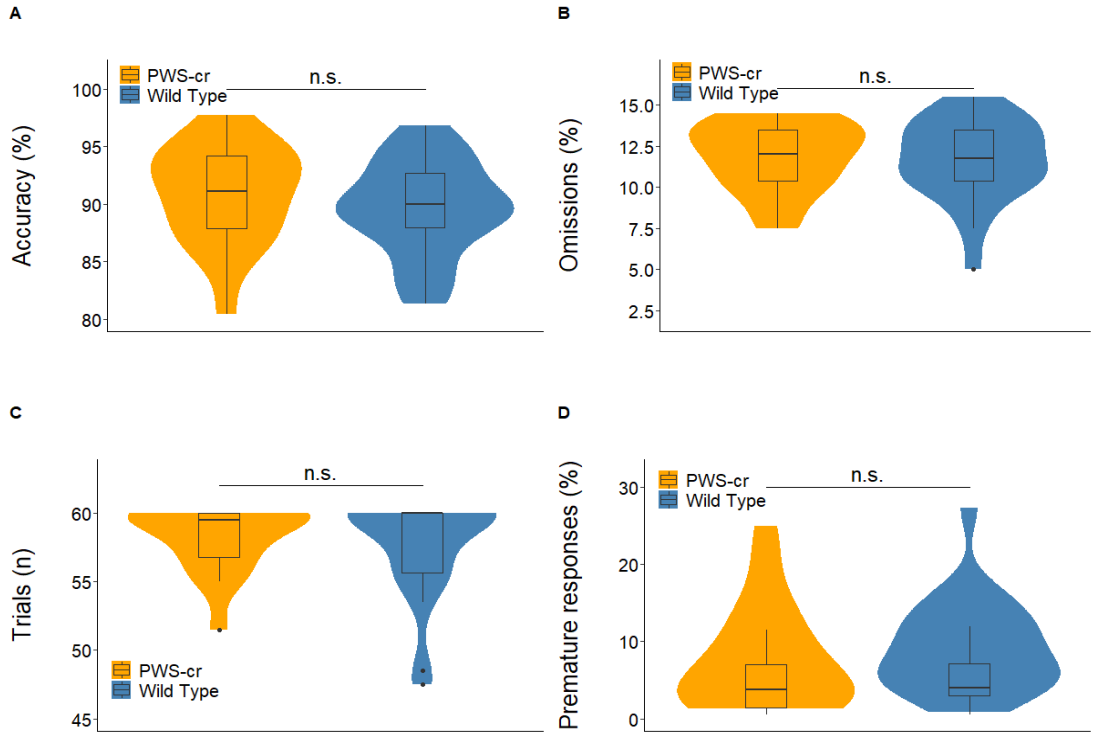


Figure 3.4 Results from baseline conditions of the 5-CSRTT. Accuracy of task performance (A), trial omissions (B), number of completed trials (C) and number of premature responses (D), all showed no significant behavioural differences between PWS-cr mice (n=20) and their wild type littermates (n=24) (p=0.724, p=0.749, p=0.925 and p=0.385, respectively).

PWS-cr mice demonstrated more rapid responding than their wild type littermates, as indexed by the correct response latency parameter (Figure 3.5A; $F_{(3, 40)}$=1.924, p=0.036), which could be indicative of a higher visuo-spatial attentional capacity. However, given that eight variables were examined in this test, a Bonferroni correction for multiple testing would lower the threshold of significance to 0.006, which changes the interpretation of the Correct Response Latency p-value to suggest no effect of genotype on that variable.

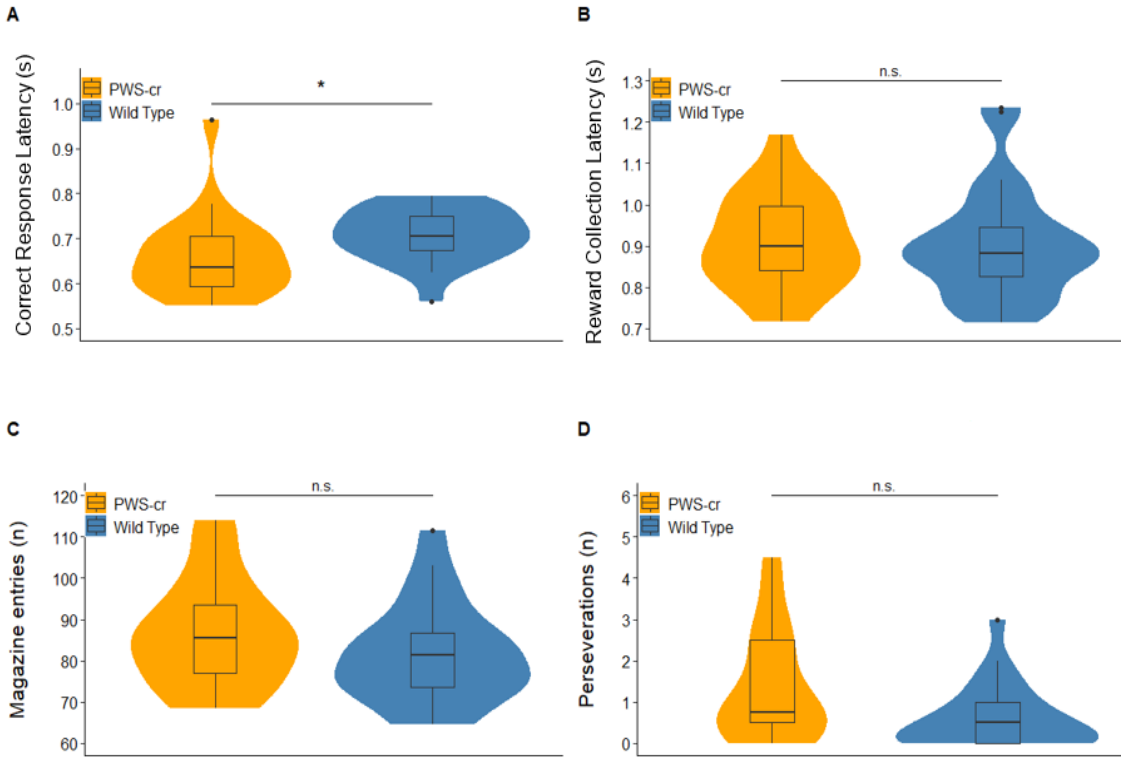


Figure 3.5 Performance at baseline conditions of the 5-CSRTT. PWS-cr mice made quicker responses in correct trials than their wild type littermates (A, p=0.036), but other parameters such as the reward collection latency (B), number of food magazine entries (C), and number of perseverative responses D) showed no differences between PWS-cr mice (n=20) and wild type littermates (n=24) (p=0.125, p=0.653 and p=0.125, respectively). The * symbol indicates p<0.05.

There was no significant difference in number of sessions taken to reach baseline conditions between PWS-cr (mean = 98.6 ±7.331 SEM) and wild type (mean = 90.833 ±5.66 SEM) (Figure 3.6A; $F_{(3, 40)}$=0.703). While there was no effect of genotype on number of training sessions taken to reach baseline or in performance at baseline conditions of the 5-CSRTT, progress through the tasks showed some differences between PWS-cr and wild type. The average number of sessions taken to get back to stable baseline after each manipulation was affected by sex, and there was a significant interaction between sex and genotype (Figure 3.6B; $F_{(3,39)}$=3.113, p=0.005,

p=0.040). The PWS-cr males, in particular, took approximately double the number of sessions on average (mean=15.171, SEM=4.699) to reach stable baseline compared to PWS-cr females, wild type males, or wild type females (mean=7.385, SEM=0.784; mean=8.700, SEM=0.922; mean=8.662, SEM=0.972). The average number of sessions taken to reach stable baseline did not differ significantly between the different manipulations ($X^2_{(4)}$=4.126, p=0.389). These results indicate that the PWS-cr interval might have a sex-dependent effect on memory or behavioural flexibility.

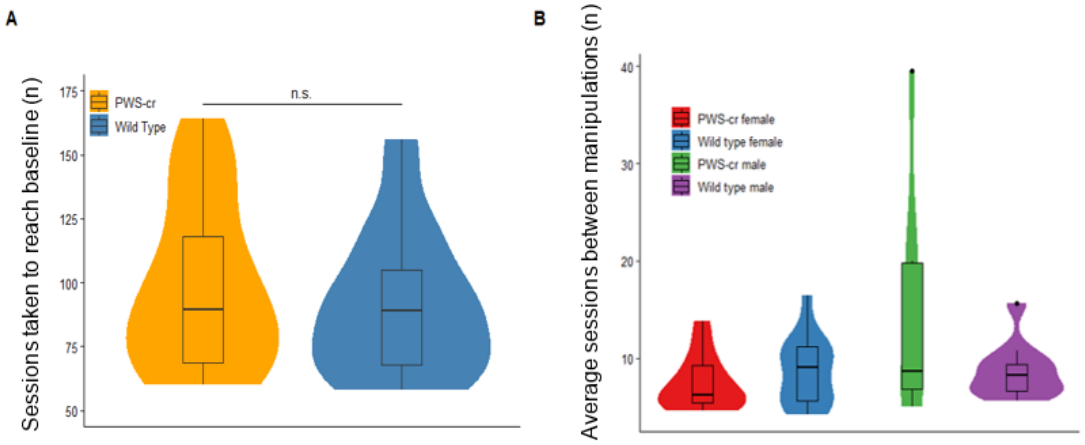


Figure 3.6 Training and progression through the manipulations of the 5-CSRTT. No difference was observed between genotypes in number of training sessions taken to reach baseline conditions (p=0.703) (A). Progression through the manipulation tasks of the 5-CSRTT calculated as average number of sessions taken to reach stable baseline between the manipulations (B) was significantly affected by genotype in an interaction with sex (p=0.040), with the PWS-cr males, in particular, progressing more slowly from baseline conditions to the final task.

3.3.3 Manipulation of the inter-trial interval durations

Of the 44 mice that reached baseline criteria at the 5-CSRTT, 18 PWS-cr and 24 wild type animals progressed to this task manipulation. In this session, the ITI of different length was introduced in pseudorandom order between trials to induce premature responding, which is considered a measure of impulsivity. As expected, longer ITI durations lead to a significant increase the number of premature responses compared to the baseline ITI duration of 5 s (Figure 3.7A; $X^2_{(3)}$=31.005, p<0.001).

In terms of genotype effects, the ITI task showed no significant differences between PWS-cr mice and their wild type littermates in most of the recorded variables, including premature responses, accuracy, omissions and correct response latency (Figure 3.7; $X^2_{(2)}$=5.834, p=0.067; $X^2_{(2)}$=0.119, p=0.945; $X^2_{(2)}$=0.526, p=0.768; $X^2_{(8)}$=8.7218, p=0.366).

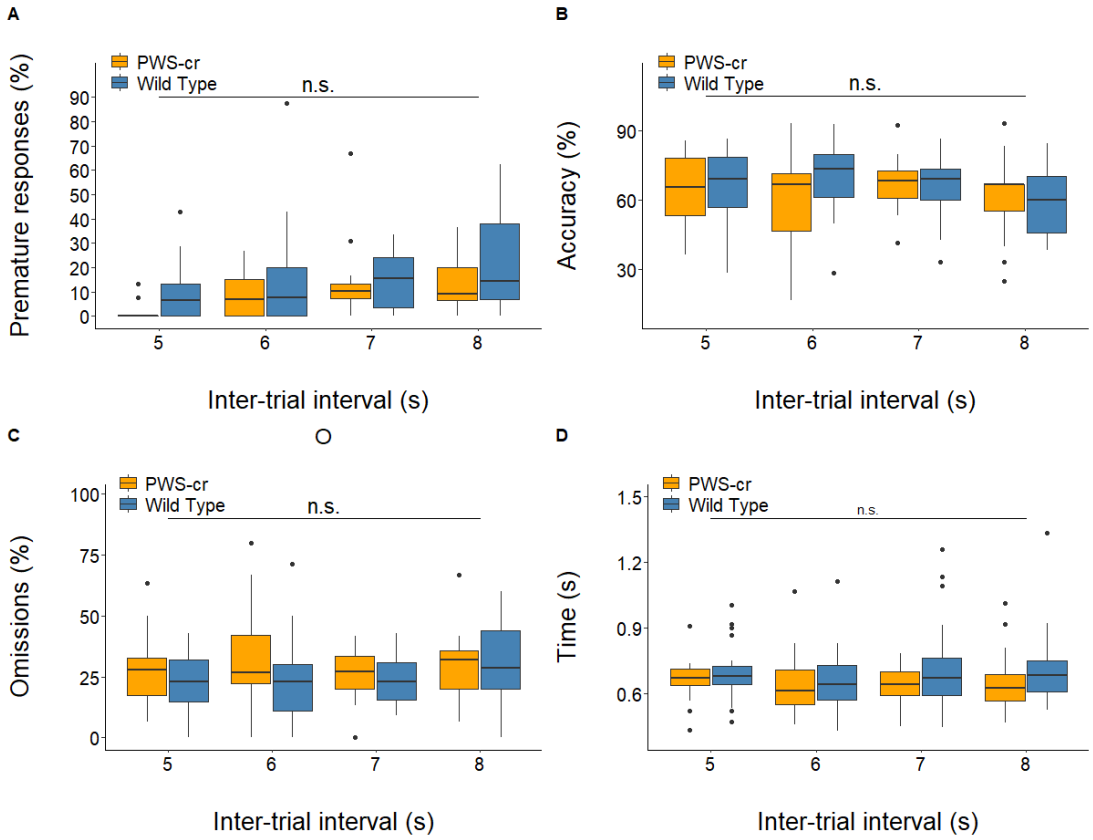


Figure 3.7 Performance with increased inter-trial interval durations of the 5-CSRTT. No difference of behaviour was observed between PWS-cr mice (n=18) and their wild type littermates (n=24) in number of premature responses (A), task accuracy (B), omission of trials (C) and correct response latency (D) (p=0.26, p=0.945, p=0.768 and p=0.366, respectively). All significance indicatives on the figure show differences between genotypes.

3.3.4 Manipulations of stimulus duration

The length of stimulus duration was manipulated with the aim to tease out differences in accuracy of task performance. The shortened stimulus duration (SSD) task pseudo randomly interspersed the baseline signal of 0.8 s, with reduced signals of 0.6, 0.4 and 0.2 s in order to increase the difficulty of the task, and highlight potentially subtler attentional phenotypes that were not evident at baseline conditions. As intended, this task reduced the accuracy of performance of all animals compared to baseline (Figure 3.8A; $X^2_{(6)}=4.905$, $p<0.001$), and increased trial omissions (Figure 3.8C; $X^2_{(6)}=23.878$, $p<0.001$). There was no effects of genotype on accuracy, correct response latency, omissions or premature responses (Figure 3.8; $X^2_{(4)}=3.489$, $p=0.48$; $X^2_{(4)}=0.986$, $p=0.912$; $X^2_{(4)}=2.067$, $p=0.723$; $X^2_{(4)}=1.429$, $p=0.489$).

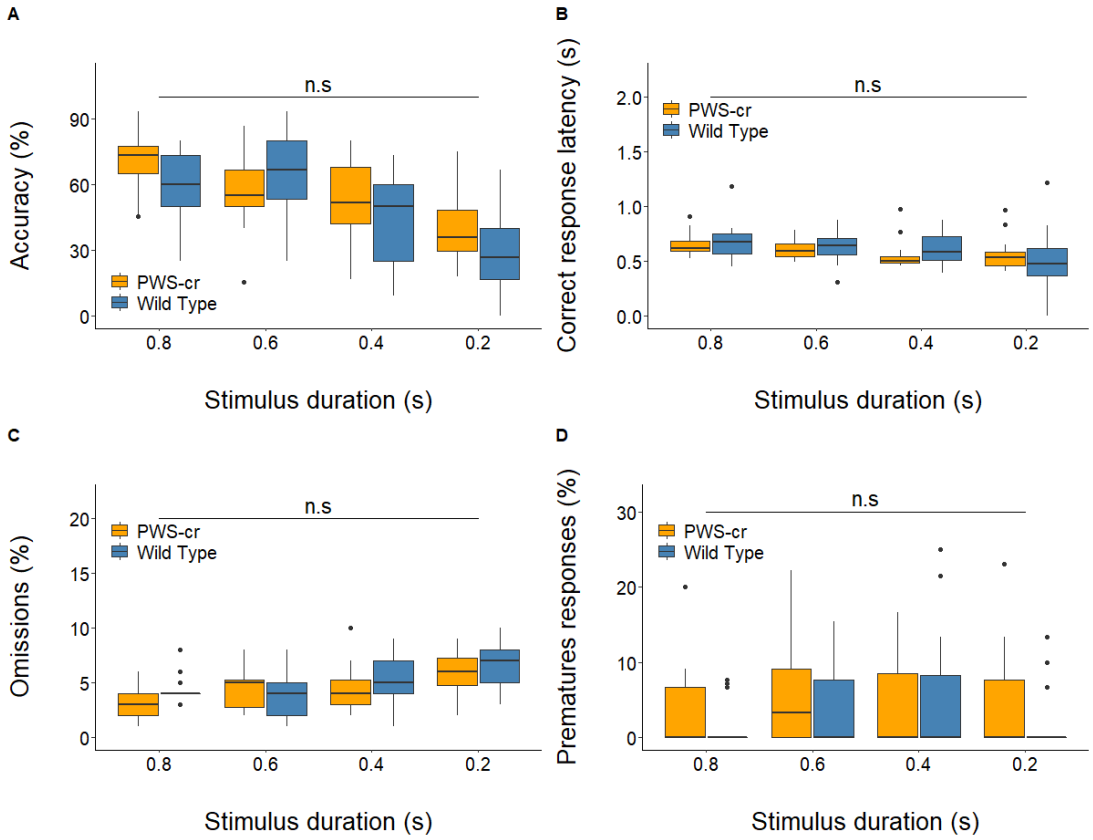


Figure 3.8 Performance at reduced stimulus duration of the 5-CSRTT. No difference of behaviour was observed between PWS-cr mice (n=16) and their wild type littermates (n=21) in task accuracy (A), correct response latency (B), omission of trials (C) and number of premature responses (D) (p=0.48, p=0.912, p=0.723 and p=0.489, respectively). All significance indicatives on the figure show differences between genotypes.

In the longer stimulus duration task (LSD), stimuli of increasing length were pseudorandomly interspersed with baseline duration in order to discern whether the PWS-cr deletion would have an effect when attentional load was decreased. This manipulation significantly decreased percentage of omissions and correct response latency (Figure 3.9C, 3.9B; $X^2_{(3)}$=23.153, p<0.001; $X^2_{(12)}$=30.693, p=0.002), but there was no effect of genotype on accuracy, correct response time, omissions, premature responses (Figure 3.9; $X^2_{(2)}$=0.951, p=0.621; $X^2_{(8)}$=5.396, p=0.715; $X^2_{(2)}$=4.347, p=0.114; $X^2_{(8)}$=2.348, p=0.872).

Overall, the results from manipulating the stimulus duration replicate the observations collected from the performance at baseline conditions of the 5-CSRTT and reinforce the implication that the deletion of the PWS-cr interval does not play a role in visuo-spatial attention.

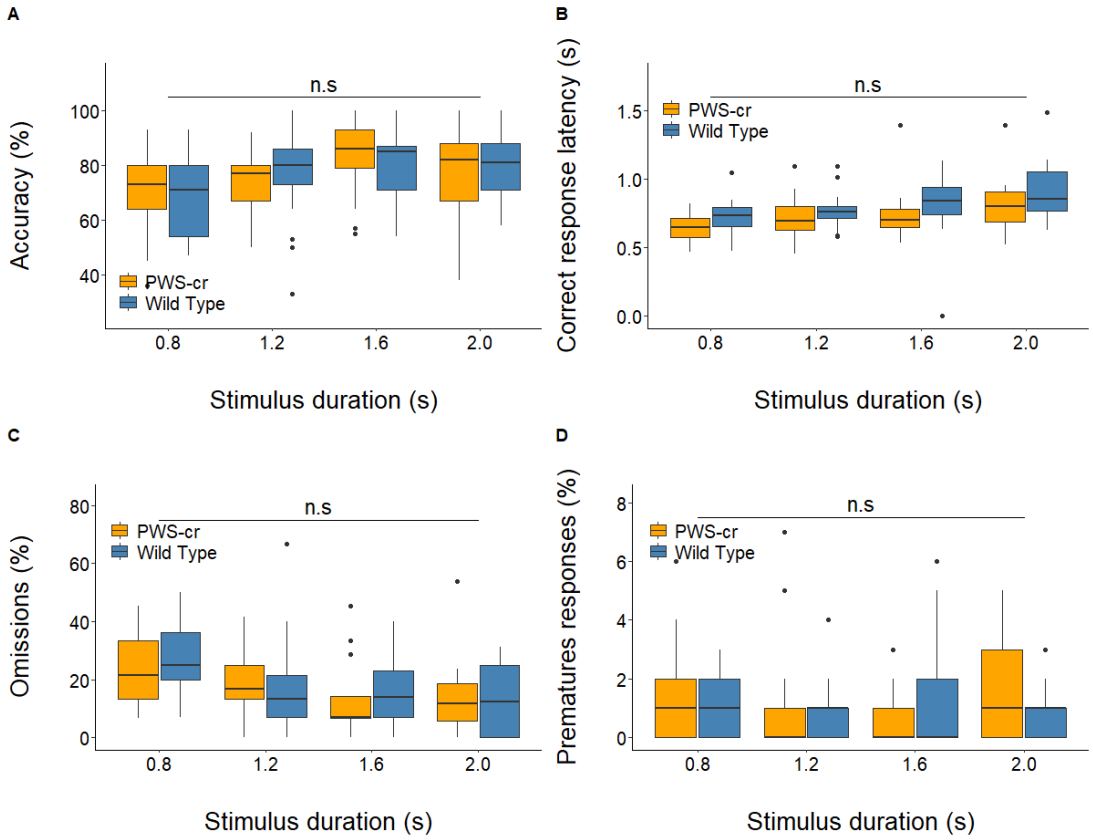


Figure 3.9 Performance at longer stimulus duration of the 5-CSRTT. No difference of behaviour was observed between PWS-cr mice (n=18) and their wild type littermates (n=24) in task accuracy (A), correct response latency (B), omission of trials (C) and number of premature responses (G) (p=0.621, p=0.715, p=0.114, p=0.872). All significance indicatives on the figure show differences between genotypes.

3.3.5 Examination of reward motivation

In order to study the motivation for the reward, mice were given free access to the reward for 10 min immediately before the start of a 5-CSRTT baseline session. Overall, the procedure did not affect the accuracy of performance (Figure 3.10A; $X^2_{(4)}$=1.439, p=0.837). However, prior access to condensed milk increased the percentage of omissions (Figure 8B; $X^2_{(4)}$=12.027, p=0.018) and reduced the number of trials (Figure 3.10C; $X^2_{(4)}$=19.508, p<0.001), which suggests that the mice were likely less motivated to perform the task due to satiety. Crucially, genotype did not have an effect on performance across accuracy, omissions, number of trials (Figure 3.10; $X^2_{(4)}$=0.846, p=0.932; $X^2_{(4)}$=2.652, p=0.618; $X^2_{(4)}$=2.203, p=0.698) or any of the measured variables after access to the reward, thus excluding hunger phenotypes as a confounding factor on performance.

Access to water was provided in the same manner as the condensed milk dilution on a different day and session, in order to discern whether the motivation to perform was driven by the reward itself, or by thirst from the water deprivation regime. The performance trends observed in this session mimicked those from the condensed milk pre-feeding session; accuracy was not affected (Figure 3.10A; $X^2_{(1)}$=0.093, p=0.760, but omissions increased significantly (Figure 8B; $X_{(2)}$=6.54, p=0.011) and the number of trials was reduced (Figure 3.10C; $X^2_{(1)}$=9.245, p=0.002), without an effect of genotype on performance in any of these three parameters (Figure 3.10; $X^2_{(1)}$=0.005, p=0.941; $X^2_{(2)}$=1.986, p=0.370; $X^2_{(1)}$=0.157, p=0.692). Notably, the same parameters showed no significant difference in performance after access to water versus performance after access to the condensed milk reward ($X^2_{(4)}$=1.334, p=0.856; $X^2_{(1)}$=1.039, p=0.308; $X^2_{(4)}$=2.638, p=0.62), which suggests that thirst is likely a key motivator for task performance rather than an interest towards the reward alone.

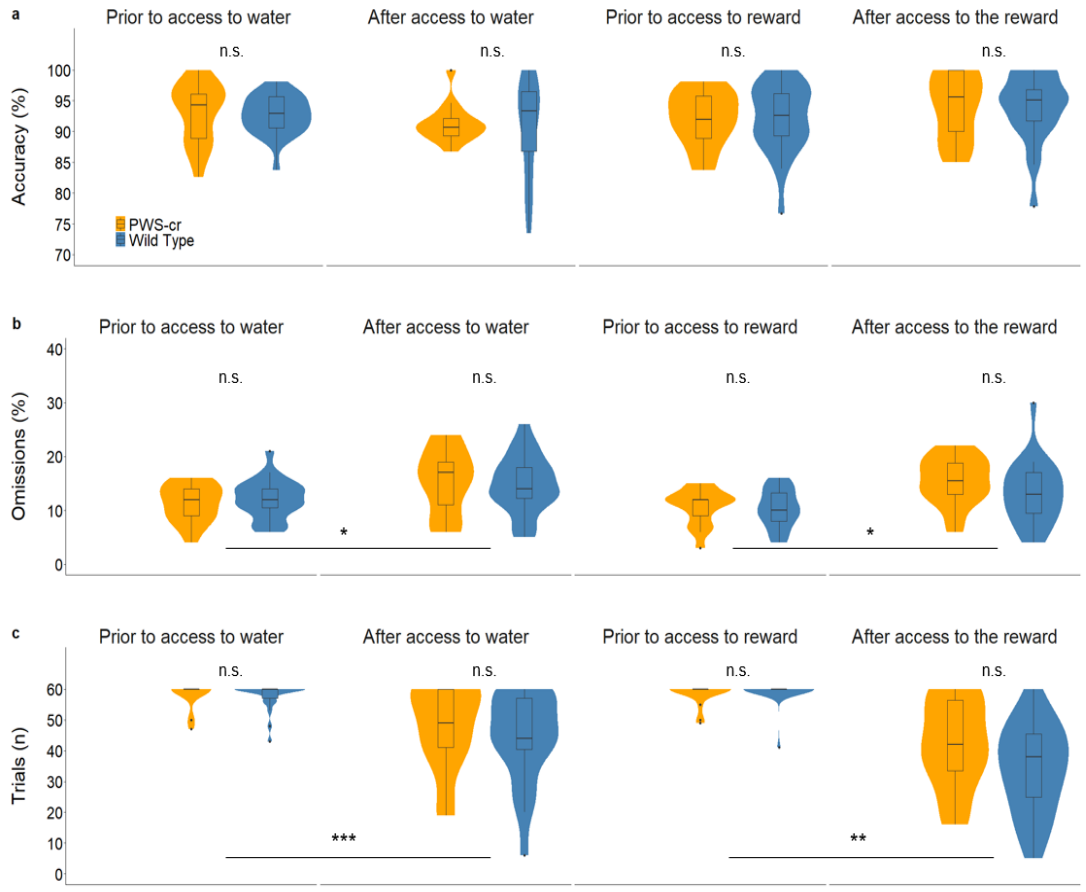


Figure 3.10 Performance at the 5-CSRTT after exposure to water or the reward. Access to water or the reward prior to a 5-CSRTT session at baseline conditions did not affect accuracy (A) (p=0.837, p=0.760), but significantly increased omissions (B) (p=0.018, p=0.011) and reduced number of trials (C) (p<0.001, p=0.002) compared to the baseline session immediately prior. Genotype did not have an effect on performance in any of the above manipulations. The standard conditions visualised in this graph are based on the average data from the two baseline session that took place immediately before the exposure to water and condense milk tasks. * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001.

3.4 Discussion

This chapter describes the behaviour of the PWS-cr mouse model in the 5-CSRTT, a task designed for studying attention and impulsivity. The PWS-cr deletion only had very subtle effects on the behaviours studied by this test. At baseline conditions of the task the PWS-cr mice made quicker responses in correct trials than their wild type littermates, but showed no other changes in behaviour in all the other examined parameters. Manipulation of stimulus duration showed no differences between the PWS-cr mice and their wild type littermates. Analysis of the average number of sessions taken to reach stable baseline after each manipulation highlighted a sex-related phenotype, which would require further study in order to draw any conclusions. Crucially, a detailed look at the data combined with two manipulations of satiety shows that the observed results are likely not confounded by motivation for the reward.

3.4.1 No differences between genotypes in performance at baseline conditions of the 5-CSRTT

Analysis of behaviour in the 5-CSRTT under baseline conditions revealed a subtle difference between PWS-cr mice and their wild type litter mates in the correct response latency variable, with the PWS-cr mice having a quicker response to visual stimuli than wild type, which might indicate that the PWS-cr deletion leads to improved attention. However, there was no statistical significance after correction for multiple testing is applied.

Overall, these results indicate that the deletion of the PWS-cr interval does not lead to any major deficiencies in visuo-spatial attention. These data differ substantially from the data collected with the same methodology of the PWS-IC “full” mouse model of PWS which has a loss of expression of the PWS-cr interval along with a few other genes from the PWS locus (Relkovic et al. 2010). In this study, PWS-IC mice showed significantly reduced accuracy, omitted a higher percentage of trials and had a reduced correct response time compared to wild type littermates (Relkovic et al. 2010), thus recapitulating phenotypes of inattention characteristic of individuals with PWS. The comparison between this study and the current results suggests that the major deficits in attention observed in the full mouse model are not likely to be caused by the loss of expression of the PWS-cr interval.

No indications of impulsivity phenotype in the PWS-cr mice in the increased stimulus duration manipulation

Increasing the ITIs induced an increase in premature responses, which are measured as an indication of impulsivity (Robbins 2002), in both PWS-cr mice and wild type. There were no differences between the genotypes, and a breakdown of the data by the length of the ITI did not yield a significant interaction in premature responses or any of the other parameters. Similarly, PWS-IC mice also showed no difference in premature responding compared to their wild type littermates in the 5-CSRTT (Relkovic et al. 2010). This consistency in findings demonstrates that impulsivity, as measured in the 5CSRTT, was unaffected by the PWS-related mutations. However, using a more specific assessment of impulsivity with the stop-signal reaction time-task (SSRTT), Davies et al. (2019) found an impulsivity phenotype in PWS-IC, suggesting that this model recapitulates some of the symptomology of PWS patients. It would be of interest to pursue this phenotype further in the PWS-cr model by assessment with the SSRTT before fully ruling out an impulsivity phenotype.

3.4.2 The PWS-cr deletion did not affect performance when stimulus duration was manipulated

The shortening and lengthening of the stimulus duration was used to increase and reduce attentional load and further assess attention in the PWS-cr mice. These manipulations did not pull out any further differences between the genotypes, thus reaffirming the original conclusion that the PWS-cr interval does not play a role in attentional phenotypes. In contrast to the PWS-cr mouse model, when presented with the reduced stimulus manipulation, the PWS-IC model bred on the same background exhibited significantly reduced accuracy of performance and increased percentage of omissions compared to their wild type littermates (Relkovic et al. 2010), consistent with their baseline differences. Thus, the data gathered from this manipulation here are consistent with the baseline performance and further suggest that loss of expression of the PWS critical interval does not contribute to the attentional phenotype seen in PWS-IC mice or individuals with PWS.

3.4.3 The PWS-cr deletion might have a subtle effect on behavioural flexibility

Number of sessions taken to reach baseline conditions was approximately the same between PWS-cr mice and their wild type littermates, whereas the PWS-IC mice exhibited some difficulties in the training process and took significantly more sessions than their wild type littermates to make it to baseline (Relkovic et al. 2010). However, there was a sex-dependent genotype effect on progressions through the

manipulations, as shown by the average number of sessions taken to reach stable baseline after each manipulation. Manipulations generally lead to a drop in performance at the following baseline sessions, possibly because the changes in conditions lead to the mice unlearning baseline performance. The male PWS-cr mice took approximately double the number of sessions that all the other groups did to reach stable baseline and move on to the next task. The meaning of these results is difficult to interpret since the 5-CSRTT is not standardly designed to study behavioural flexibility or unlearning phenotypes. Furthermore, none of the other analysed variables at the 5-CSRTT exhibited any interactions between sex and genotype. Since the PWS-cr male group had half the n of the other groups, it is possible that the interaction between sex and genotype is a random effect of low statistical power.

A study of the PWS-cr mice reported no preference towards novel objects which could be a result of inflexible behaviour, but the authors of the paper attributed it to impaired location memory and sex did not have an effect on the observed phenotypes (Adhikari et al. 2019). There are no other studies in literature that examine behavioural flexibility in this mouse model, so further investigation would be required before any conclusions can be drawn. A reversal learning task could be used in the future to assess whether this mouse model has a sex-specific effect on behavioural flexibility.

3.4.4 The PWS-cr deletion did not affect reward motivation

The hyperphagia phenotype in the PWS-cr mouse model had to be taken into account since it could act as a confounding factor in a study that relies on food-based reward delivery to motivate performance. While some studies have previously reported hyperphagic behaviour typical of individuals with PWS in the PWS-cr model (Ding et al. 2008; Qi et al. 2016b), other studies have shown no differences in the feeding behaviour of the deletion-carriers compared to wild type (Powell et al. 2013; Polex-Wolf et al. 2018). The data from this chapter would support the latter view, with no differences in feeding behaviour between PWS-cr and wild type mice, as demonstrated in the reward preference test. Here, PWS-cr mice did not consume more condensed milk than wild type, nor did they show an increased preference towards the condensed milk reward. Secondly, their performance of the 5-CSRTT under baseline conditions did not show patterns resembling changes in motivational state, as standardly examined by number of completed trials, number of food tray entries or reward collection latency (Robbins 2002).

Finally, a manipulation that focused particularly on the role of reward motivation was implemented to test this further. Mice were given access to either water or the

condensed milk reward before the start of a 5-CSRTT baseline session, in order to inspect whether satiety would affect the performance of PWS-cr mice differently to that of their wild type littermates. As expected, these manipulations reduced the motivation for participation in the task in both groups (reduced completed trials) but the results did not differ by genotype. There were no differences in the effect of this manipulation when the mice were pre-fed with either water of the reward, suggesting that the mice were likely more driven by thirst caused by the restricted water regime rather than by motivation for the palatable and more rewarding condensed milk. Collectively, these observations reject the idea that hyperphagic phenotypes could be influencing the results of the 5-CSRTT.

3.4.5 Conclusion

The results from the experiment presented in this chapter indicate that the PWS-cr mice do not display the same attention and impulsivity deficits seen in the PWS-IC model using identical apparatus. This suggests that the critical interval does not play a role in the attention and impulsivity deficits that are highly prevalent in individuals with PWS. The elimination of *SNORD116* and *IPW* as potential causes of ADHD-like behaviours in PWS is an important step towards establishing the genetic and molecular roots behind these phenotypes. Furthermore, this study contributes to the characterisation of the behavioural profiles of PWS mouse models and eliminates the PWS-cr model in particular as a platform for pre-clinical screening of drugs targeting attention and impulsivity.

Chapter 4. Transcriptomic study of neonatal brain tissue from the PWS-cr and PWS-IC mice

4.1 Introduction

The results from the behavioural study outlined in Chapters 2 and 3 demonstrated a vastly different behavioural profile in the PWS-cr mouse model compared to the previously examined PWS-IC mouse model. The PWS-IC mice exhibited a wide variety of behavioural phenotypes of relevance to ADHD and psychotic disorders (Relkovic et al. 2010; Davies et al. 2019), whereas in the behaviours examined in this thesis, the PWS-cr mice exhibited only subtle differences in behaviour compared to control, that could potentially be indicative of affective disorders. To investigate the transcriptomic differences guiding the distinct behavioural profiles of PWS-cr and PWS-IC mice, I performed a transcriptomic study looking at differentially expressed genes and isoforms in whole brain tissue from each of these mouse models and their wild type littermates.

While all behavioural tasks were conducted on adult individuals, neonatal mouse tissue was used for the RNA-sequencing assay due to an availability of PWS-IC whole brain samples at foetal and neonatal stages from previous work with this model. Crucially, the imprinted genes in the PWS locus are expressed in the mouse brain at birth (Lee et al. 2003; Yamasaki et al. 2003; Skryabin et al. 2007) and the examined behaviours are likely to be affected by early brain development. Genetic risk for cognitive and psychiatric phenotypes manifests from early developmental stages (Mistry et al. 2018; Schork et al. 2019) and organizational neuronal processes of relevance to psychiatric illness have been shown to take place in the neonatal brain (Garza et al. 2018).

The psychiatric and cognitive disorders examined by this thesis have all been linked to neurodevelopmental disturbances. Neuroimaging studies of childhood ADHD, as observed in individuals with PWS, have shown it to be a result of impaired brain development (Valera et al. 2007; Arnsten and Rubia 2012; Friedman and Rapoport 2015). These data are further solidified by genomic studies, which show that genetic variants of relevance to ADHD are preferentially associated with neurodevelopmental genes (Elia et al. 2010; Poelmans et al. 2011).

From the psychiatric illnesses, schizophrenia has been robustly shown to be strongly rooted in neurodevelopment. The “neurodevelopmental model” for schizophrenia suggests that although in most patients symptoms begin to manifest in adulthood, it is early brain development that is responsible for the pathology of the disease, and

genetic mechanisms of schizophrenia might converge with those of brain development (Weinberger 1987; Lewis and Levitt 2002; Rapoport et al. 2005). This model is supported by genomic studies which have discovered that genetic variants of relevance to schizophrenia are also associated with neurodevelopmental disorders such as autism spectrum disorder, intellectual disability and ADHD (Girirajan and Eichler 2010; Girirajan et al. 2012; Grayton et al. 2012; Ellis et al. 2016; Birnbaum and Weinberger 2017). There is neuroimaging and genomic evidence to suggest that major psychosis, major depressive disorder (MDD) and bipolar disorder (BP) are also linked to early neural development (Ansorge et al. 2007; Chen et al. 2014; Xiao et al. 2014; Zhao et al. 2015; Gałecki and Talarowska 2018; Schmitgen et al. 2019).

The regulatory role of the critical interval has not been examined in the neonatal mouse brain, but studies of adult mice have shown it to affect gene expression (Qi et al. 2016b; Coulson et al. 2018), with Coulson et al's study in particular showing a role of the PWS-cr in rhythmicity of DNA methylation. The individual roles and molecular targets of the genes from the critical interval are not entirely clear, but a handful of studies in cell culture have shown that *SNORD116* regulates the expression of genes related to hunger and circadian rhythms (Falaleeva et al. 2015; Polex-Wolf et al. 2018), while *IPW* regulates the expression of a cluster of imprinted micro RNAs in the *DLK1-DIO3* gene locus on chromosome 14 (Stelzer et al. 2014), which are linked to hypotonia (Kagami et al. 2017). Furthermore, snoRNAs have been suggested to play a role in splicing regulation (Kishore and Stamm 2006a) and *Snord115* from the PWS locus, in particular, has been discovered to regulate the alternative splicing of the 2C serotonin receptor (Kishore and Stamm 2006b; Raabe et al. 2019). Therefore, the transcriptomic study included analysis of differential isoform usage in order to examine whether *Snord116* has a similar role in splicing, which in combination with its effect on gene expression levels could be contributing to the behaviour profile of the PWS-cr mouse.

The PWS-IC mouse is expected to exhibit a dysregulation of over a dozen genes, including the two genes of the critical interval. The disruption of the entire PWS locus is likely to have a multitude of downstream effects, which could be contributing to behavioural phenotypes of the PWS-IC model and could explain why so many more behaviours are affected in it than in the PWS-cr model. With this study, I hope to examine the transcriptomic differences between the neonatal brains of the two mouse models, and to identify some of the molecular bases for the psychiatric illness associated with the two genotypes.

4.2 Methods

4.2.1 Tissue collection and RNA extraction

Whole brain tissue from PWS-IC mice and their wild type littermates was collected at stages E13.5, E18.5, and P0 between 10:00 am and 12:00 pm, snap frozen in dry ice and stored at -80°C until further use. The tissue was homogenized in Matrix D tubes (MP Biomedicals, UK) in an appropriate volume of TRI reagent (Sigma, UK). 1 ml of Trizol was added per 50-100 mg of samples. After 5 min incubation at room temperature for 5 min, 0.2 ml of chloroform was added to each reaction, gently mixed and incubated again at room temperature for 2-3 min, before being centrifuged at 4000 rpm and 8°C for 15 min. The aqueous phase was transferred to a fresh tube and had 0.5 ml of isopropanol added per reaction before being incubated at room temperature for 10 min and then centrifuged at 4000 rpm at 8°C for 10 min. Supernatant was discarded and the RNA pellet washed with 1 ml of 75% ethanol per reaction, then vortexed briefly and centrifuged again at 4000 rpm at 8°C. The RNA pellet was air dried and resuspended in an appropriate volume of DEPC-treated MilliQ water and incubated at 60°C for 10 minutes. The extracted RNA was then cleaned up using the RNeasy mini protocol (Qiagen). This collection and extraction was performed by Dr. Jennifer Davies.

Whole brain tissue from PWS-cr and their wild type littermates was collected at P0, snap frozen in dry ice and stored at -80°C until further use. The tissue was homogenized in Matrix D tubes (MP Biomedicals, UK) in an appropriate volume of TRI reagent (Sigma, UK). RNA was extracted using the Direct-zol RNA Miniprep kit following the provided protocol.

4.2.2 RT-qPCR

In preparation for the RT-qPCR, 1 µg of each sample's RNA was reverse transcribed into cDNA with the EcoDry Premix double primed kit (Clontech), following the provided protocol. cDNA was diluted 1:10 in nuclease-free water and stored at -20 °C until further use. Primers for *Snord116, Snord115, Necdin, Mkrn3, Magel2* and *Ube3a* were used as targets of investigation. *Hprt, Gadph* and *B2m* were used for housekeeping genes as a positive control. A reaction of 25 µL was prepared by adding 1.75 µL of each primer, 12.5 µL of 2X SensiMixSYBR No-ROX (Bioline), 5 µL of the sample and 4 µL of nuclease free water. Nuclease free water was added instead of a sample in each PCR run as a negative control. All samples and controls were ran in triplicates. The PCR was performed on a Corbet Rotor 30 Gene 6000 Real-Time PCR machine. The cycling conditions used for each reaction were: **1)** 95°C

10 minutes, **2)** 95°C 20 secs, **3)** 60°C 20 secs, **4)** 72°C 20 secs, repeat cycles 2-4 40 times, **5)** 1°C increment increase from 50 to 99°C in order to generate melt curves, which were subsequently inspected to check that each primer pair was generating only one product.

The resulting data were averaged across the triplicates for each sample. ΔCt values were calculated by normalising expression data to the positive control — subtracting the geometric mean of the housekeeping genes from the Ct value for each target gene in each sample. ΔCt values for each condition were used for statistical analysis. Average ΔCt for wild type samples was subtracted from average ΔCt of each condition in order to calculate ΔΔCt.

Table 4.1 RT-qPCR primer sequences for target and housekeeping genes.

Gene	Primer sequence	Concentration
Snord116	ATCTAATGATGATTCCCAGTCAAACAT	300 nM
	TCACTCATTTTGTTCAGCTTTTCC	300 nM
Snord115	ACAACCCACTGTCATGAAGAAAGG	50 nM
	CCTCAGCGTAATCCTATTGAGCAT	900 nM
Necdin	ATGGTGCAGAAGCATCCTCAG	300 nM
	ATGGTGTGGAGATTGGTCAGC	300 nM
Mkrn3	CCAATCAGTTGCTTAAGAAGTTGC	300 nM
	AAGAGCCAACGGTCATCAGAG	300 nM
Magel2	GCATAGCAAGCCAGCCTCAG	300 nM
	GTAGACGAGCCTGTGGAGCCT	50 nM
Ube3a	CAGACGTGACCATATTATAGATGATGC	700 nM
	CCACATACAACTGCTTCTTCAAGTCT	700 nM
Hprt	GCGATGATGAACCAGGTTATGA	300 nM
	GCCTCCCATCTCCTTCATGA	300 nM
Gadph	GAACATCATCCCTGCATCCA	300 nM
	CCAGTGAGCTTCCCGTTCA	300 nM
B2m	TTCTGGTGCTTGTCTCACTGA	300 nM
	CAGTATGTTCGGCTTCCCATTC	300 nM

4.2.3 RNA sequencing

Libraries for RNA-sequencing were prepared with the KAPA mRNA HyperPrep kit (Roche Sequencing solutions) from total RNA of RQN > 8.5 as assessed by the Fragment Analyser system (Agilent Technologies). 800 ng of polyA selected RNA

was used for each sample for a paired and unstranded sequencing approach. The target read depth was 80 mln reads per sample. The mRNA was fragmented at 94°C for 6 minutes to achieve a mean insert size of 200-300 bp. 8 cycles were used in the final amplification. A final bead clean-up step was added at the end of the protocol before the libraries were taken forward for NGS on an Illumina HiSeq4000 system with approximately 80 million reads. Quality control, library preparation and RNA-sequencing were performed by Joanne Morgan.

4.2.4 RNA sequencing analysis

The heatmap.2 package in R was used for hierarchical clustering. For analysis of differential gene expression, sequencing reads were mapped to the mouse genome (version mm10) using the STAR software package (Dobin and Gingeras 2015). The featureCounts software (Liao et al. 2014) was used to count the raw reads. The difference between wild type and the PWS mouse models were subsequently analysed in R Studio 1.1.383 using the DESeq2 package (Anders and Huber 2010), with sex and litter as covariates. The UCSC genome browser was used for visualization of the data. Differential isoform usage was pseudo-aligned to the mouse transcriptome (mm10) with the Kallisto software (Bray et al. 2016) which was also used to quantify the raw reads. The Kallisto files were then analysed in R studio via DEXSeq with the IsoformSwitchAnalyzeR package (Anders et al. 2012; Ritchie et al. 2015; Vitting-Seerup and Sandelin 2017), for genotype differences with sex and litter added as covariates. All p-values were adjusted using the Benjamini-Hochberg method. Prediction of protein domains was run on EBI's Pfam webserver, which performs biosequence analysis using hidden Markov models (Punta et al. 2011). The data from the PWS-cr and PWS-IC models was analysed separately since difference between the wild type littermates of the two groups did not allow for pooling of the data. Genes that were differentially expressed or exhibited differential usage of isoforms between genotypes (padj<0.05) were pooled together for gene ontology analysis using the g:Profiler web server (Raudvere et al. 2019).

4.2.5 Common variant enrichment analysis

Genes that were differentially expressed or exhibited differential usage of isotopes between genotypes (padj<0.05) were pooled together for common variant enrichment analysis. The mouse gene IDs were converted to their homologous human gene IDs. The genes were run for gene set analysis through MAGMA along with GWAS summary statistics for schizophrenia, psychosis and chronic kidney disease, which was used as negative control since individuals with PWS haven't been reported to

exhibit any problems with kidney function (Pardiñas et al. 2018; Legge et al. 2019; Wuttke et al. 2019). Gene locations build 37 and reference data from European population was downloaded from CNCR CTGLab.

4.3 Results

4.3.1 Gene expression profile of PWS genes at different developmental stages

PWS-IC whole brain samples at stages E13.5, E18.5 and P0 were available for the transcriptomic study. In order to select which tissue samples to use for this investigation, RT-qPCR was used to quantify the expression of the genes at the PWS locus across the three stages. The results of the qPCR validated the expected gene expression of the PWS-IC mouse model. With the exception of *Magel2* and *Mkrn3,* which did not come across as expressed in any of the examined samples, the PEGs showed loss of expression in PWS-IC tissue compared to wild type, while the MEG *Ube3a* was overexpressed (Figure 4.1). At stage E13.5 only *Necdin* showed a significant difference in expression ($t_{(8)}$ = -3.407, p-value = 0.009), while *Snord115, Snord116,* and *Ube3a* were not differentially expressed between the different genotypes ($t_{(8)}$ = -2.002, p = 0.08; $t_{(8)}$ = -1.7145, p = 0.1248; $t_{(8)}$ = 0.5013, p = 0.630). At stage E18.5 *Snord115, Snord116, Necdin* and *Ube3a* all showed significantly differential gene expression between the PWS-IC samples and wild type ($t_{(6)}$ = 11.788, p < 0.001; $t_{(6)}$ = -6.415, p < 0.001; $t_{(6)}$ = -24.033, p < 0.001; $t_{(6)}$ = -12.956, p < 0.001), as well as at stage P0 ($t_{(8)}$ = -3.573, p = 0.007; $t_{(8)}$ = -6.297, p < 0.001; $t_{(8)}$ = -7.4468, p < 0.001; $t_{(8)}$ = -6.415, p = 0.033). It is important to note that while some of the genes showed no expression in some samples, it's possible that some expression is highly localised and therefore lost in examination of whole brain tissue samples.

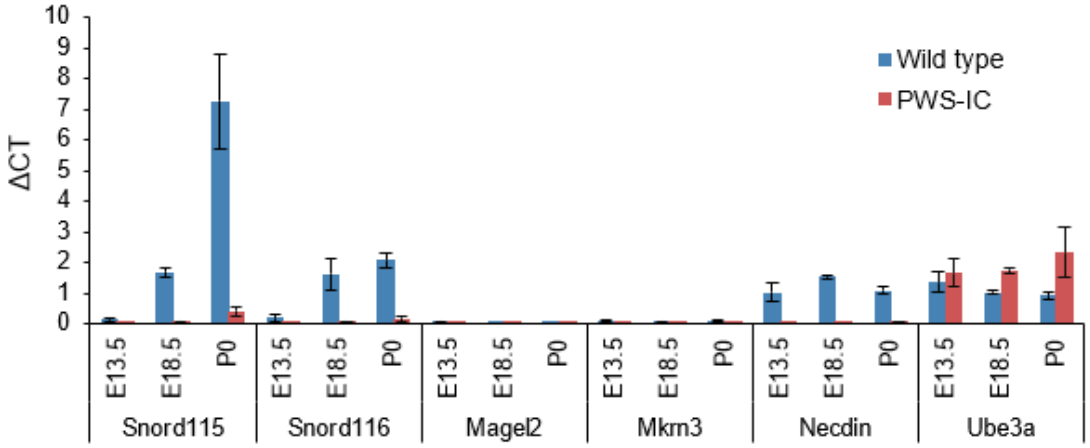


Figure 4.1 Expression of the genes from the PWS locus in whole brain tissue at two developmental and one postnatal stage. RT-qPCR data validated the expression of the PWS-IC model, by showing the expected loss of expression of the PEGs *Snord115, Snord116* and *Necdin,* and an overexpression of the MEG *Ube3a*, particularly at 18.5E stage of foetal mouse development and at birth (bars indicate mean, error bars indicate SEM).

Out of the PWS locus, the *Ube3a* gene has been most strongly linked to psychiatric illness in literature (Soni et al. 2008; Yang et al. 2013; Noor et al. 2015; Salminen et al. 2019), so to capture potential transcriptomic changes of relevance to psychiatric illness in the PWS-IC model, it was essential to examine samples where *Ube3a* was dysregulated compared to wild type. The expression of *Snord115* was also taken into consideration because the snoRNA has been previously suggested to regulate the expression of the 2c serotonin receptor which plays a role in some of the studied behaviours (Nonogaki et al. 2003; Morabito et al. 2010; Garfield et al. 2016; Davies et al. 2019). Finally,, the expression of *Snord116* was taken into account since the gene was a main target of investigation in the PWS-cr mouse model. Based on this, only stages E18.5 and P0 were considered for the transcriptomic study because at E13.5 *Snord115* and *Snord116* were not expressed, while *Ube3a* did not show differential expression between PWS-IC and wild type. Due to the higher facility of obtaining samples at P0 and the higher number of samples available from the PWS-IC model at P0, the neonatal stage was selected for an RNA-sequencing study.

4.3.2 RNA-sequencing analysis

PWS-IC heterozygous mice (4 female) and 6 wild type littermates (2 female, 4 male) as well as 6 PWS-cr heterozygous mice (3 female, 3 male) and 6 wild type littermates (3 male, 3 female) were used for the RNA-sequencing assay. Hierarchical clustering showed a female wild type outlier in both PWS-cr and PWS-IC controls, which were removed from further analysis (Figure 4.2).

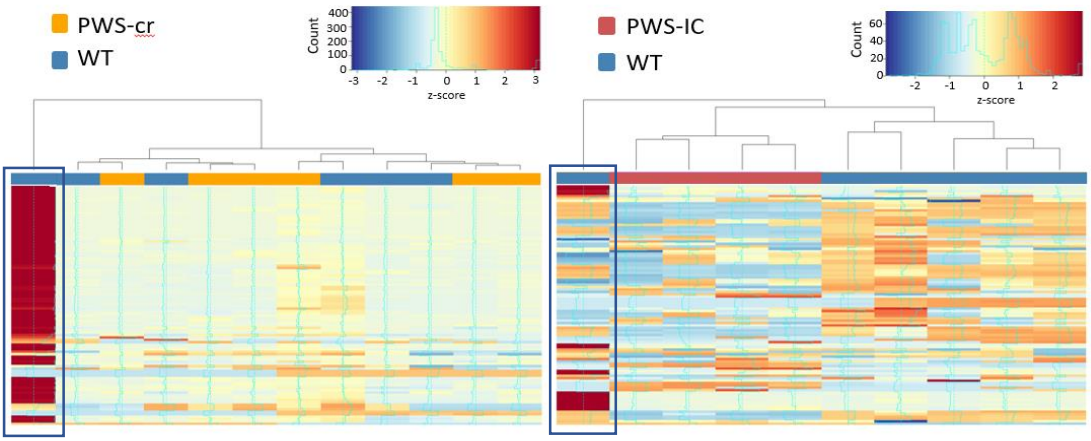


Figure 4.2 Hierarchical clustering heat maps based on the top 100 differentially expressed genes in the PWS-cr and PWS-IC neonatal mouse brain samples. The PWS-cr samples had few differentially expressed genes and did not cluster by genotype (A; columns represent samples, rows represent genes). In contrast the PWS-IC samples exhibited more significant expression differences compared to their controls, as reflected by the samples clustering by genotype. A an outlier for the wild type control of both models (B; far left on each heatmap) was excluded from further analysis. The blue line in each sample indicates raw read counts.

Furthermore, PWS-IC samples clustered by genotype, whereas PWS-cr samples did not, which was also observed in PCA analysis (Supplementary figure 1).

4.3.3 Differentially expressed genes and isoforms in the PWS-cr mouse model

Visualization of the aligned RNA-sequencing reads in the UCSC browser confirmed that the deletion in the PWS-cr mouse model mode spans all copies of Snord116 snoRNA and five of the six *Ipw* exons (Figure 4.3a). However, differential gene expression analysis showed only very subtle differences between the PWS-cr mice and their wild type littermates after Benjamini-Hochberg adjustment for multiple testing.

In total, there were seven differentially expressed genes (DEGs) in neonatal brain tissue from the PWS-cr mouse model, and only three of known function (Figure 4.3b). Among them was the *Snhg14* non-coding RNA, which acts as a host for the critical interval transcripts (z=21.846, padj<0.001). The *Mafa* gene encoding a transcription factor that regulates pancreatic beta cell-specific expression of the insulin gene was upregulated in the absence of the critical interval (z=-7.014, padj<0.001). Notably, the *Necdin* growth suppressor which is one of the imprinted genes of the PWS locus, was upregulated in the PWS-cr mouse brain tissue (z=-8.621, padj<0.001). The remaining four DEGs were predicted genes of unknown function, including *Gm44831* which falls into the critical interval.

In addition to differential gene expression, the read depth used in the RNA-seq analysis allowed analysis of isoform switches with functional consequences in order to investigate whether the genes in the critical interval play a regulative role in alternate splicing. Seven genes showed significantly different isoform usage in the PWS-cr mouse brain, and there was no overlap between them and the DEGs. The differentially expressed genes and isoforms were pooled together for a gene ontology analysis, which revealed no enrichments.

Interestingly, among the differentially expressed isoforms was the *dual*-specificity tyrosine-phosphorylation-regulated kinase (*Dyrk3*), which plays a role in the dissolution of stress granules and in the early secretory pathway (Wippich et al. 2013), and works in synergy with *Dirk1a,* which has been linked to intellectual disability (Courcet et al. 2012a; Ji et al. 2015; Luco et al. 2016). The differential isoform use data shows that the wild type mouse model brain tissue contains a truncated isoform of *Dyrk3* (Figure 4.3c), which is lacking in the PWS-cr samples. This isoform is missing multiple functional kinase domains (Figure 4.3d) some of which were

unspecified by the Pfam webserver, while others were recognised as tyrosine phosphorylases.

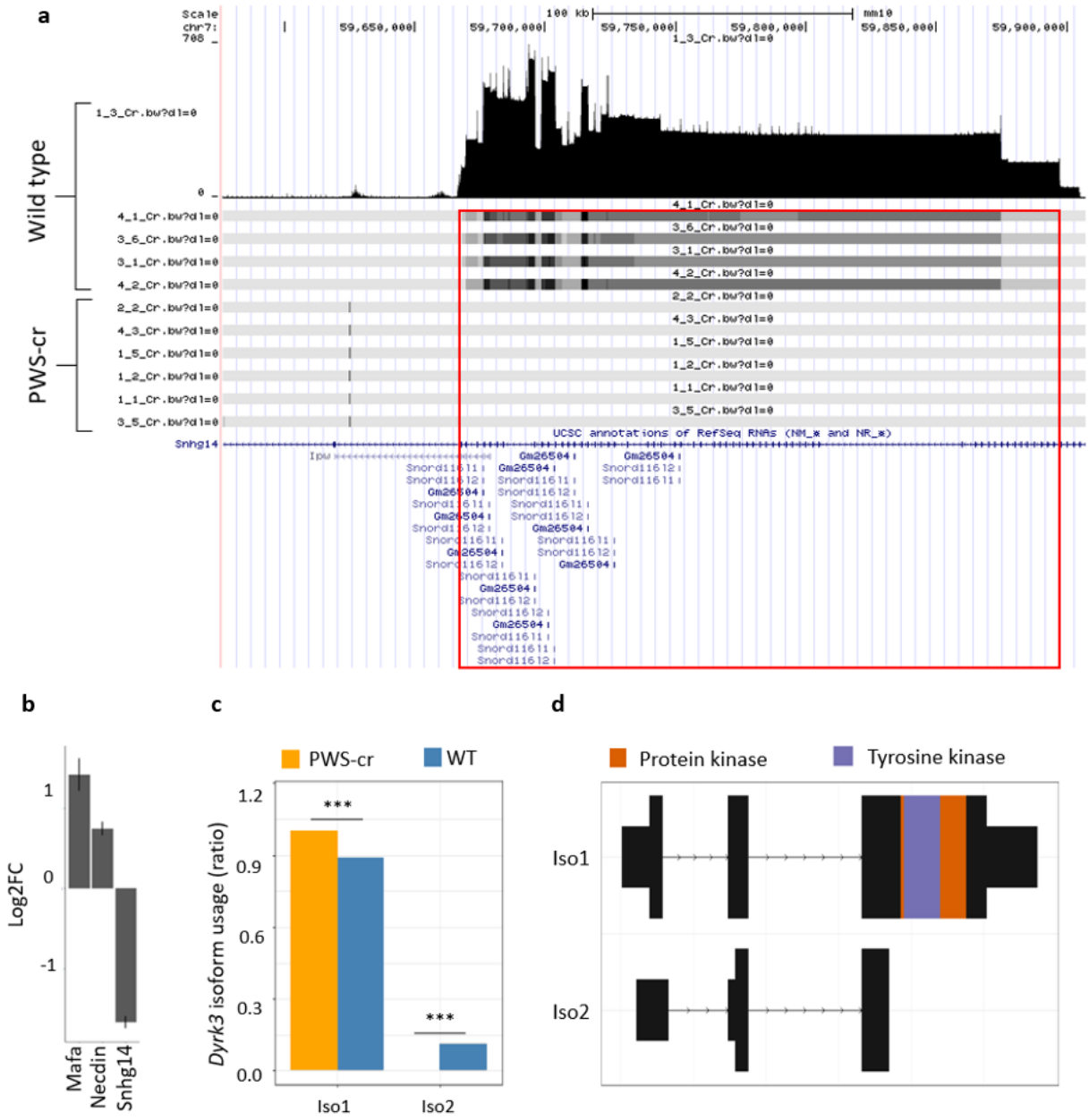


Figure 4.3 Differential gene expression and isoform use in the neonatal PWS-cr mouse brain. Transcript count data visualised with the UCSC browser (a) showed the deletion in the PWS-cr mice spans all the copies of *Snord116,* as well as most of the exons of the *Ipw*. There were three significant differences in gene expression (b); *Necdin* and *Mafa* were overexpressed in PWS-cr tissue, while the expression of *Snhg14* was lost. The y axis on this graph (b) shows log2 fold change of expression, error bars indicate SEM. Analysis of differential isoform usage showed an isoform of *Dyrk3* lacking tyrosine and protein kinase domains (d - Iso2) in the wild type mice, which was absent in the PWS-cr mice (c).

4.3.4 Differentially expressed genes and isoforms in the PWS-IC mouse model

Analysis of the PWS-IC neonatal mouse brain revealed 59 significant DEGs compared to their wild-type littermates, 28 of which were of known function (Figure 4.4a). This included *Snhg14*, the non-coding RNA that hosts *Snord116* and *Ipw*, but also, as expected, the other PWS genes such as *Snrpn*, *Necdin*, *Mkrn3*, and *Magel2*. Outside the PWS cluster there were other notable DEGs, including the circadian clock regulator *Per1* (z=-4.25, padj=0.012) and insulin growth factor *Igf1* (z=4.164, padj=0.015).

Analysis of isoform switches identified 48 differential isoform uses, and there was no overlap with the differentially expressed genes. Among them was the GABA receptor subunit gene *Gabrg3*, which is associated with autism and Angelman syndrome, a sister disorder to PWS caused by mutations in the same cluster of imprinted genes (Greger et al. 1995; Wang et al. 2018). Furthermore, the differential isoform usage data shows that the PWS-IC samples exhibit increased usage of a truncated isoform of *Gabrg3* (Figure 4.4b) which is missing neurotransmitter-gated ion-channel ligand binding domains and neurotransmitter-gated ion-channel transmembrane region (Figure 4.4c).

Differentially expressed genes and isoforms were pooled together for a gene ontology term analysis (n=105), which were enriched for molecular functions, cellular compartments and biological compartments relevant to oxygen transport (Table 4.2).

***Table* 4.*2* Enriched gene ontology terms of differentially expressed genes and isoforms.**

Gene ontology class	Term name	GO ID	N genes	Adjusted p-value
Molecular function	haptoglobin binding	GO:0031720	3	0.000702
Molecular function	oxygen carrier activity	GO:0005344	3	0.00292
Molecular function	hemoglobin alpha binding	GO:0031721	2	0.008323
Molecular function	hemoglobin beta binding	GO:0031722	2	0.008323
Molecular function	oxygen binding	GO:0019825	3	0.027568
Biological process	oxygen transport	GO:0015671	3	0.017211
Cellular compartment	hemoglobin complex	GO:0005833	3	0.000425
Cellular compartment	haptoglobin-hemoglobin complex	GO:0031838	3	0.000425

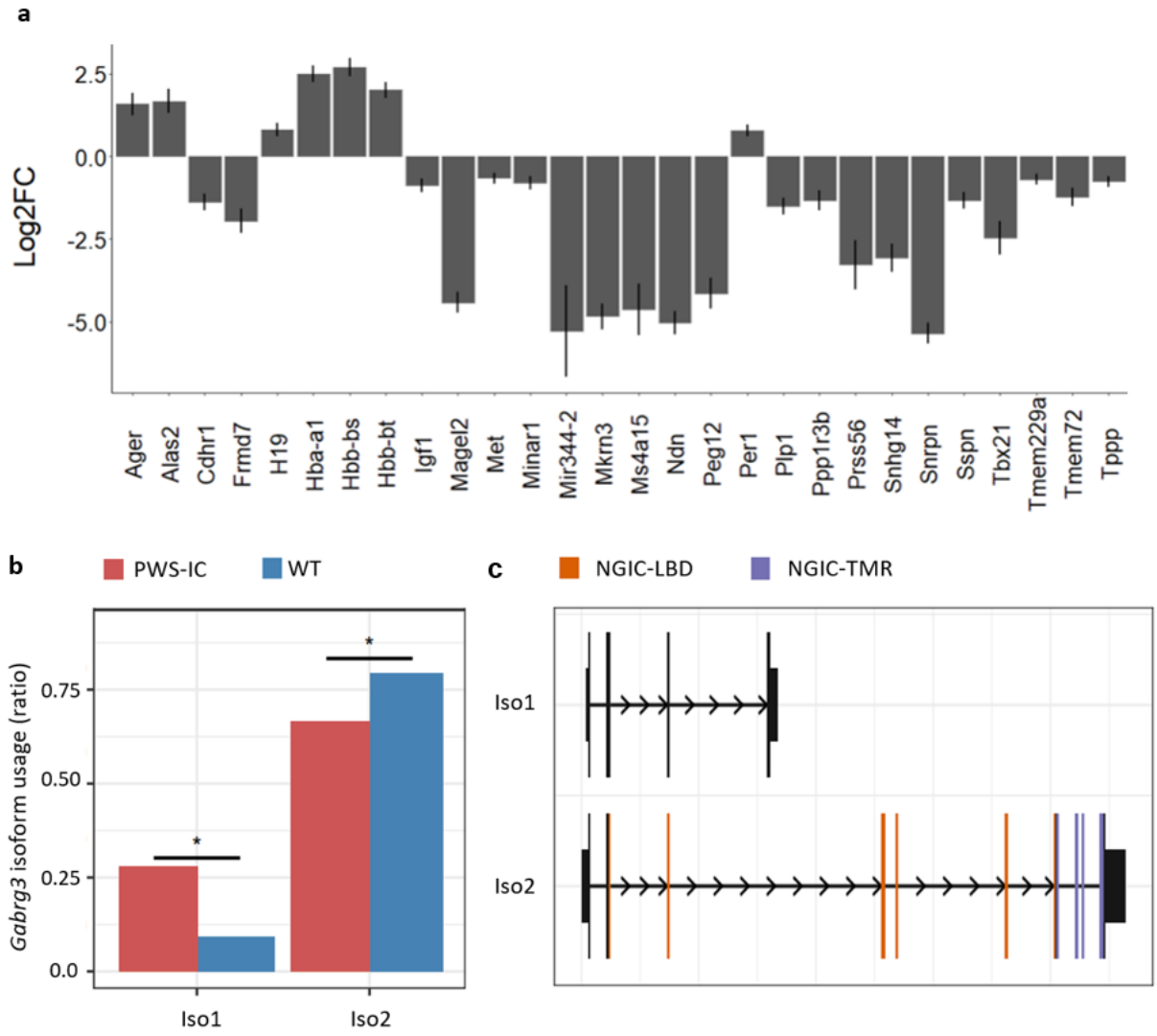


Figure 4.4 Differential gene expression and isoform use in the neonatal PWS-IC mouse brain. Among the differentially expressed genes in the PWS-IC tissue were the genes from the PWS locus, and notably the circadian clock regulator Per1 and the insulin growth factor *Igf1*. Predicted genes of unknown function were excluded from the graph. The y axis on this graph (a) shows log2 fold change of expression, error bars indicate SEM. Analysis of differential isoform usage showed increase use (b) of a truncated isoform of *Gabrg3* lacking neurotransmitter-gated ion-channel ligand binding domain (NGIC-LBD) and neurotransmitter-gated ion-channel transmembrane region (NGIC-TMR) (c – labelled as Iso1) in the PWS-IC samples.

A major issue to be addressed was the imbalanced sex groups between genotypes in the PWS-IC mouse model. All the PWS-IC samples were female, which can confound the results and could potentially mean that some of the observed transcriptomic differences were a product of sex differences. A look at the differences in the neonatal whole brain tissue from the PWS-cr mouse model analysed by sex, showed a total of 16 DEGs (Supplementary Figure 2) and 13 differentially used isoforms (Supplementary Table 1), among which were key markers of sex

differences, including *X-inactive specific transcript (Xist)*, and Ubiquitously Transcribed Tetratricopeptide Repeat Containing, Y-Linked (*Uty)*. Overall, none of the transcriptomic changes analysed by sex overlapped with the transcriptomic changes observed in the PWS-IC mice.

4.3.5 Enrichment of common genetic variants

Comparing the combined DEG and isoform data for the two models directly shows that there were a greater number of differences in the PWS-IC mice overall (Figure 4.5a). As expected, a good proportion (~50%) of the changes seen in PWS-cr were shared with those found in PWS-IC. However, this overlap was limited to the DEGs, as there were no common differentially spliced genes in the PWS-cr and PWS-IC samples.

The differentially expressed genes and differentially used isoforms (padj<0.05) were pooled together for each mouse model in order to look for enrichment of common genetic variants associated with schizophrenia or psychotic episodes. The analysis was performed using MAGMA's gene-set computation with the datasets from genome wide association studies (GWAS) that utilize the UK Biobank, ClozUK and CKDgen consortium databases respectively (Pardiñas et al. 2018; Legge et al. 2019; Wuttke et al. 2019). The results showed no enrichment of genetic variants of interest in the PWS-cr samples (Figure 4.5b). In contrast, results from the PWS-IC model indicated an enrichment of genes common for the experience of 'any' and 'multiple' psychotic episodes (β=0.215, p=0.023, β=0.271, p=0.007), but interestingly not for schizophrenia (β=-0.206, p=0.913). As expected, neither DEG and isoform list was enriched for the negative control dataset, common genetic variants associated with chronic kidney disease (β=-0.161, p=0.909).

Furthermore, a closer inspection of the individual genes that carry common genetic variants of psychotic episodes shows that among them was *Gabrg3* and, interestingly, the PWS PEGs *Mkrn3, Magel2,* and *Necdin*. These findings suggest a cumulative effect of several of the genes from the PWS locus on the psychotic illness typical of Prader-Willi syndrome.

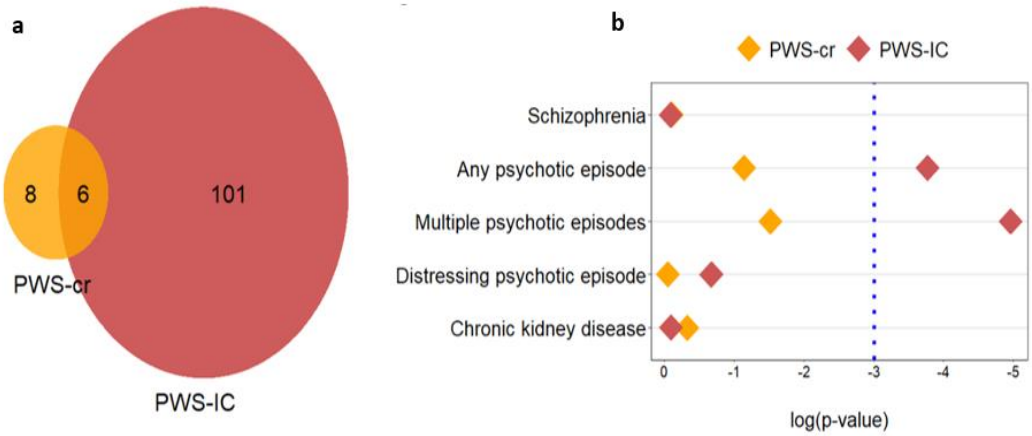


Figure 4.5 Enrichment analysis for common variants of schizophrenia and psychotic episodes. Differentially expressed genes and isoforms from the neonatal brain samples of the PWS-cr and PWS-IC mouse models were pooled for enrichment analyses; the two models had 6 genes in common (a). Gene set analysis of pooled differentially expressed genes and isoforms (b) showed a significant enrichment of common genetic variants associated with the experience of psychotic episodes, but not schizophrenia. GWAS summary data from a chronic kidney disease study was used as a negative control.

4.4 Discussion

This chapter explores the transcriptomic bases for the behavioural differences between PWS-cr and PWS-IC mouse models using an RNA-sequencing assay on whole brain neonatal samples. The study of the PWS-cr tissue showed only subtle changes in gene expression and isoform usage that, albeit individually interesting, were not enriched for any specific phenotype. Although this study found no evidence of the PWS-cr interval having a role in psychiatric illness, there is some indication that its deletion might have an effect on cognition.

As to be expected, the PWS-IC mice exhibited a greater difference in transcriptomic profile than PWS-cr mice when compared to their respective controls, since they present a dysregulation of the entire PWS locus including the PWS-cr interval. Furthermore, the transcriptomic changes in the PWS-IC brain are enriched for common genetic variants associated with the experience of psychotic episodes, but not schizophrenia. These findings might shed a light on the nature of psychotic illness in PWS.

4.4.1 Expression profile of PWS-IC model was validated at P0 stage of development

An RT-qPCR study of expression levels of the PWS locus genes from whole brain tissue at developmental stages E13.5, E18.5 and P0 validated the expected gene expression of the PWS-IC mouse model. At E18.5 and P0 the majority of the PEGs with the exception of *Mkrn3* and *Magel2* showed loss of expression in PWS-IC tissue compared to wild type, while the MEG *Ube3a* was overexpressed, and this profile was not present or not as pronounced in the E13.5 samples, which informed the decision to use E18.5 or P0 samples for the transcriptomic analysis. Due to the ease of collection and the higher n of neonatal samples, P0 was selected for the assay.

4.4.2 Transcriptomic effects of the PWS-cr deletion

Analysis of RNA-seq data from the PWS-cr mice revealed only seven differentially expressed genes and five differential isoform uses when compared to wild type. Previous studies have demonstrated that PWS-cr has an effect on the expression of upwards of 200 genes (Stelzer et al. 2014; Falaleeva et al. 2015; Polex-Wolf et al. 2018). However, these studies have looked at gene expression in specific tissues or cell types, whereas the study here presents a crude look at the changes occurring in whole brain tissue, which likely led to the obscuring of some of the subtler or more

localized outcomes and showed only the strongest effects. No molecular or functional enrichments were found in the differentially expressed and spliced genes.

4.4.3 Necdin *expression is altered in PWS-cr mice*

The data from the RNA-seq study suggests that PWS-cr acts, either directly or indirectly, as a suppressor of *Necdin. Necdin* is an important regulator of neuronal outgrowth and differentiation (Lee et al. 2005; Kuwajima et al. 2006) and its loss in mice is associated with motor deficits and enhanced learning and memory (Muscatelli et al. 2000; Andrieu et al. 2006). Interestingly, a loss of the critical interval in a mouse model has been shown to lead to deficits in learning and memory as measured by the novel object recognition paradigm (Adhikari et al. 2019). It is therefore possible that the cognitive impairment observed in the PWS-cr mouse model is due, in part, to the overexpression of *Necdin.* Furthermore, the correlation between PWS-cr and *Necdin* stacks on to piling evidence of imprinted genes regulating each other with other prominent examples being the regulatory loop between *Snord116* and *Snord115* (Falaleeva et al. 2015) and the suppressive role of *IPW* on the maternally expressed genes from the *DLK1-DIO3* imprinted cluster (Stelzer et al. 2014). Understanding the mechanics of this regulatory relationship between imprinted genes could potentially be revealing of the role of genomic imprinting.

Necdin is not only expressed in the mouse brain throughout development, but also throughout adulthood (Uetsuki et al. 1996; Niinobe et al. 2000). In future studies it would be interesting to explore whether the alteration in *Necdin* expression is present in the adult PWS-cr mouse brain as well.

4.4.4 Differential isoform usage of Dyrk3 *in PWS-cr mice*

Another interesting effect of the PWS-cr deletion was the loss of a truncated isoform of *Dyrk3*, which lacks the transcripts for functional kinase domains, including a tyrosine phosphorylase. The expression levels of the gene were not different between PWS-cr mice and their wild type littermates, but the use of a truncated isoform could potentially inhibit the reactions *Dyrk3* is involved with by binding its molecular targets without being able to complete its kinase function. Furthermore, *Dyrk3* auto-phosphorylates the tyrosine on its own activation loop, which plays an important part in its kinase activity (Kim et al. 2018), so the truncated form could be affecting the function of the longer isoform as well. It is possible that this truncated isoform serves

as a way of downregulating the activity of *Dyrk3* in wild type tissue and its absence in the PWS-cr leads to over-activation of the gene.

DYRK1A, a far more studied gene from the same molecular family has been directly implicated in microcephaly, intellectual disability and speech impairment (Courcet et al. 2012a; Ji et al. 2015; Luco et al. 2016). Although *DYRK3* has not been directly linked to intellectual disability or any related cognitive phenotypes, it has been shown to work in synergy with *DYRK1A* in the phosphorylation and activation of Sirtuin1 (SIRT1) deacetylase, which has also been linked with intellectual disability (Guo et al. 2010; Dalal et al. 2019). While the behavioural study in this thesis did not cover learning and memory, it is possible that *Dyrk3* contributes to the cognitive deficit phenotypes described by Adhikari et al's (2019) novel object recognition experiments. Crucially, *Dyrk3* not only has a role in protein modification, but also is a key regulator of the early secretory pathway (Whippich et al. 2013) and has a role in the proteome which would not be detected by a transcriptomic study such as this one.

Notably, the truncated version of *Dyrk3* was discovered only in the PWS-cr samples and not in the PWS-IC ones, which also lack an expression of the critical interval. It is possible that the dysregulation of the entire PWS imprinted locus in the PWS-IC mice leads to the loss of interactions that play a role in the truncation of *Dyrk3.* This discovery is in congruence with the distinct difference of phenotypes between PWS individuals with delPWS genetic subtype versus those that carry mUPD15. While the latter group has a more burdensome load of symptoms and a higher incidence of psychosis in particular, they tend to have a significantly higher IQ than individuals carrying deletions of the paternally expressed genes on chromosome 15 (Roof et al. 2000; Whittington et al. 2004). For further studies of the role of the PWS-cr on cognition, I would conduct a more in-depth study looking into behaviours related to aspects of learning and memory in the PWS-cr mouse, such as the y maze and novel object recognition task. Given a confirmation of the current results, the individual roles of *Ipw* and *Snord116* within the critical region should also be investigated, including their molecular targets, and the pathways they are involved with.

4.4.5 Transcriptomic effects of the PWS-IC deletion

In contrast to the limited changes seen in the PWS-cr model, RNA-seq analysis of PWS-IC neonatal brain tissue revealed a markedly larger number of DEGs and differential isoform usage. A gene ontology analysis revealed an enrichment for

molecular functions, cellular compartments and biological compartments relevant to oxygen transport, which plays an important role in neurogenesis among other processes, and has been linked to cognitive function and hyperglycaemia (Zhang et al. 2011; Zhang et al. 2012; Nalivaeva et al. 2018).

A notable limitation of this study is the lack of male samples with the PWS-IC deletion. Although the examination of sex differences at P0 showed no overlap in transcriptomic changes when analysed by sex versus when analysed by genotype in the PWS-IC mouse model, it is possible that the results from this model are confounded by poor representation of the sexes.

4.4.6 Reduced expression of Plp1 *and increased expression of* Per1 *in PWS-IC neonatal whole brain tissue*

Analysis of differential gene expression showed a downregulation of *Plp1* in the PWS-IC brain tissue. Disruptions in *Plp1* function have been most strongly linked to dysmyelinating disrders such as Pelizaeus-Merzbache and spastic paraplegia type 2, which are characterised by spastic quadriplegia, ataxia, mild intellectual disability and developmental delay (Inoue 2005; Lee et al. 2006; Garbern 2007). Recently, a genomic study found evidence that genetic variation in *Plp1* influences interhemispheric integration via the corpus callosum and functional hemispheric asymmetries (Ocklenburg et al. 2017), which are linked to language, face processing, emotional processing, and visuo-spatial attention (Hausmann 2005; Grimshaw and Carmel 2014; Ocklenburg et al. 2014). In a follow up study of the phenotypic effects of these variations in *Plp1,* Ocklenburg et al. (2018) confirmed a functional role for these polymorphisms in spatial attention. This suggest *Plp1* could potentially be a contributing factor to the attentional deficit phenotypes previously recorded in the PWS-IC mice (Relkovic et al. 2010).

Plp1 has been shown to carry genetic variants that confer an increased susceptibility to schizophrenia (Qin et al. 2005). Furthermore, a study of cortical tissue from schizophrenia patients showed a downregulation of *Plp1,* among other myelination genes (Tkachev et al. 2003), which suggests the reduced expression of *Plp1* observed in the PWS-IC neonatal brain samples could be contributing to the psychotic illness phenotypes observed in the adult mice and in individuals with mUPD15.

Per1, which is predominantly known as a circadian clock regulator, was also differentially expressed in the PWS-IC mouse brain. This was not too surprising, since *Necdin* and *Snord116* have both been reported to affect the expression of *Per1*

(Powell and LaSalle 2015; Lu et al. 2020). While in the PWS-IC mouse model the expression of both *Snord116* and *Necdin* is lost, in the PWS-cr mouse model, the deletion of *Snord116* leads to overexpression of *Necdin*. It is possible that this interaction between the two genes in the PWS-cr mice counterbalances in a way that neutralises the effect on *Per1* expression. Notably, *Per1* has been associated not only with circadian rhythms but also with aspects of psychiatric illness. A study by Novakova et al. (2015) reported that the expression profile of *PER1* is affected in patients with mania but not depression. Furthermore, *Per1* is regulated by cortisol, and it has been linked to stress-induced alcohol consumption (Dong et al. 2011). Therefore, it is possible that *Per1* contributes to the psychiatric profile of individuals with mUPD15.

4.4.7 Increased production of a truncated isoform of Gabrg3 in PWS-IC samples

Among the genes that exhibited differential isoform usage in the PWS-IC samples was *Gabrg3, which* exhibited an increased production of a truncated isoform of *Gabrg3* lacking neurotransmitter-gated ion-channel domains and a decreased usage of the full isoform of the gene, which collectively could lead to reduced functionality of the protein. Since the GABA receptors play a key role in the function of the central nervous system this alternate isoform usage could have an effect on various phenotypes including psychotic illness (Ahn et al. 2011; Taylor and Tso 2015). This further ties in with a study by Webb et al. (2008), which showed that in a significant proportion of individuals with delPWS that exhibit psychosis, the deletion span parts of *GABRG3*. This suggests that the GABA receptor could also be contributing to psychotic illness in individuals with the mUPD15 genotype due to reduced functionality caused by differential isoform usage. *GABRG3* has also been linked to autism and Angelman Syndrome (Aman et al. 2018; Wang et al. 2018), so it is possible that it plays a role in some of the other behaviours observed in the PWS-IC mouse model.

4.4.8 Enrichment of variants relevant to psychosis but not schizophrenia in the PWS-IC differentially expressed genes and isoforms

Another interesting discovery of this study was the enrichment of common genetic variants relevant to psychosis in the differentially regulated genes of the PWS-IC tissue samples. The GWAS data used for this study sampled individuals with a history of psychotic experiences including hallucinations and delusional ideation, but specifically without a diagnosis of schizophrenia (Legge et al. 2019). They found a

shared genetic liability with schizophrenia from an external GWAS dataset (Pardiñas et al. 2018), which were also used for this analysis in order to identify potential genetic correlation with schizophrenia. The results showed an enrichment in the PWS-IC samples of genetic variants common to the experience of psychotic episodes, but not schizophrenia. This substantiates the behavioural endophenotype links to psychotic illness observed in the PWS-IC but not the PWS-cr mice, as well as the increased incidence of psychotic type disorders observed in individuals with the mUPD15 genetic subtype. Notably, among the genes enriched for variants relevant to psychosis were the myelin protein proteolipid protein *Plp1* and the gamma-aminobutyric acid (GABA) A receptor subunit gamma 3 gene (*Gabrg3)*, as well as *Mkrn3, Magel2,* and *Necdin* which suggests a role of the PWS PEGs in psychotic illness.

4.4.9 Conclusion

In conclusion, the RNA-seq study showed only subtle effects of the PWS-cr deletion on transcriptomic profile of the neonatal mouse brain. Although its regulation of *Necdin* and *Dyrk3* suggests a potential link to cognition, this study found no evidence to suggest that PWS-cr plays a role in the psychotic illness commonly observed in individuals with PWS. Crucially, the findings on the enrichment of common variants for psychotic episodes and schizophrenia in the PWS-IC tissue samples suggests the genetics of psychosis in Prader-Willi syndrome might not overlap with the genetics of schizophrenia. This could have implications on the therapeutics that are used to treat psychosis in individuals with PWS since different psychotic disorders respond to different doses and schedules of an antipsychotic, and to different antipsychotics altogether.

Chapter 5. Analysing the effect of genetic variation within the PWS-cr interval on depression and cognition

5.1 Introduction

The results from the behavioural and transcriptomic studies collectively suggest that PWS-cr does not have a role in psychotic illness in mice, but might have a role in the intellectual disability and increased prevalence of depression observed in individuals with PWS. In order to further investigate the contribution of PWS-cr towards these phenotypes, I conducted a study examining whether the genetic variation of this region is linked to cognition and mood in humans.

Cognition includes a wide spectre of abilities involving learning, reasoning and problem solving, as well as attention, perception and memory, and is considered a predictor of success as measured by educational and career attainment, socio-economic status and health (Deary et al. 2007; Strenze 2007; Schmidt and Hunter 2016). Major depressive disorder (MDD) or depression, is also negatively correlated with the same criteria of success. MDD is one of the most common mental illnesses at a prevalence of 5-15% and is a leading cause of suicide (Bromet et al. 2011; Ferrari et al. 2013; Whiteford et al. 2015).

Both cognition and depression are highly complex traits controlled by multiple loci, some of which have already been determined through GWAS (Howard et al. 2018; Savage et al. 2018; Wray et al. 2018). Genetic variants within these loci, such as single nucleotide polymorphisms (SNPs) and copy number variations (CNVs), are the driving factors of phenotypic diversity. Complex traits are shaped by the combined effects of multiple variants, most of which individually have a small and undetectable effect on phenotype.

Three different analytical approaches were taken to examine whether there is a link between genetic variation in the critical interval and the phenotypes of interest. The first approach was to apply principal component analysis (PCA) to genotype data of the PWS-cr, which compresses the variation of each individual into a much smaller number of variants called principal components (PCs). The PCs were then tested for association with each phenotype of interest. The second approach was to use MAGMA gene analysis, which is a method of examining the joint effect of a group of variants on a trait of interest (de Leeuw et al. 2015). The final approach was to generate polygenic risk scores (PRS) for the variants in PWS-cr, which are used to estimate the susceptibility of any one individual to a particular genetic disease or trait based on the genetic variants that that individual carries. Furthermore, since the

genes in PWS-cr have been robustly demonstrated to contribute to stature and obesity (Bieth et al. 2015; Fontana et al. 2017; Polex-Wolf et al. 2018), I used the same analytical approaches to examine whether there is a link between genetic variation in PWS-cr and height, body mass index (BMI), and percentage body fat as a comparison.

The aim of this chapter is to investigate whether PWS-cr contributes to the phenotypes of cognition and depression in individuals with PWS by analysing the link, if any, between genetic variation within that region and the phenotypes of interest.

5.2 Methods

5.2.1 Genotype data

Genotype data was obtained from the UK biobank under project 13310. All genotype data underwent quality control with PLINK2.0 (Purcell et al. 2007). SNPs with P-value lower than 1e-6 from the Hardy-Weinberg Equilibrium exact test, SNPs that are missing in >1% of individuals and individuals with >1% of missing genotype data were also excluded. Highly correlated SNPs were removed by pruning in a 200 variant window, moving down the genome in increments of 50 variants and filtering SNPs with LD r^2>0.25. Samples with heterozygosity coefficient outside of 3 standard deviations of the heterozygosity coefficient mean were excluded. Rare SNPs with minor allele frequency (MAF) <0.01 were also excluded. Genotype data for PWS-cr alone was extracted with PLINK2.0 from bp 25296623 to bp 25367623 on chromosome 15 of build hg19.

5.2.2 Phenotype data

Two different definitions of depression were used for analysis, which were curated from UKBB phenotype data by Kendall at al. (2019). The first one was constructed as a binary variable which combines self-reported depression with current antidepressant prescription (SRDAP) (416 564 controls, 26 343 cases). Individuals who only fulfilled one of these two criteria were previously excluded from the data altogether (n=15 287). The second variable was a binary measure of a hospital discharge for depression (HDD) (397 415 controls, 12 607 cases). Data from assessment centres in Scotland were excluded since they did not include records from psychiatric wards.

Cognition was analysed on the basis of the results from 7 UKBB database tests, in attempt to capture and examine the different aspects of cognitive performance. The pairs matching test (PMT) was used to assess episodic memory, reaction time test (RTT) to assess simple processing speed, fluid intelligence test (FIT) to assess reasoning and problem solving, digit span test (DST) to assess numeric working memory, symbol digit substitution test (SDST) to assess shape recognition and complex processing speed, and trail making tests A and B (TMTA, TMTB) to assess visual attention. The test results were curated from UKBB data by Kendall et al. (2017).

Standing height data from the UKBB (data-field 50) was taken with a Seca 202 device. Body fat data (data-field 21002) were estimated by impedance measurement from

1% - 75% in 0.1% increments. Body mass index (BMI) (data-field 23104) were derived by dividing weight over height squared (kg/m^2). All physical measure data were taken from Instance 0 of measurements.

5.2.3 Principal component analysis

Five principal components were generated with PLINK2.0 for PWS-cr region after a scree plot evaluation (Figure 5.1). Data were analysed in linear and logistic regressions with the PWS-cr principal components used as a between-subject factor. Sex, age and age^2 were added as covariates. Up to five whole genome variation principal components (generated previously by Dr Sophie Legge) were also added as covariates to correct for population stratification (Price et al. 2006). The test results presented for the collective effect of the PWS-cr principal components were calculated by using Chi-square or F test with the ANOVA function in R to compare the statistical models to reduced models with the PWS-cr principal components removed. The reported R^2 was taken from the results of the model containing the PWS-cr principal components. Bonferroni correction was applied to correct for multiple testing, adjusting the p-value threshold for significance to 0.004.

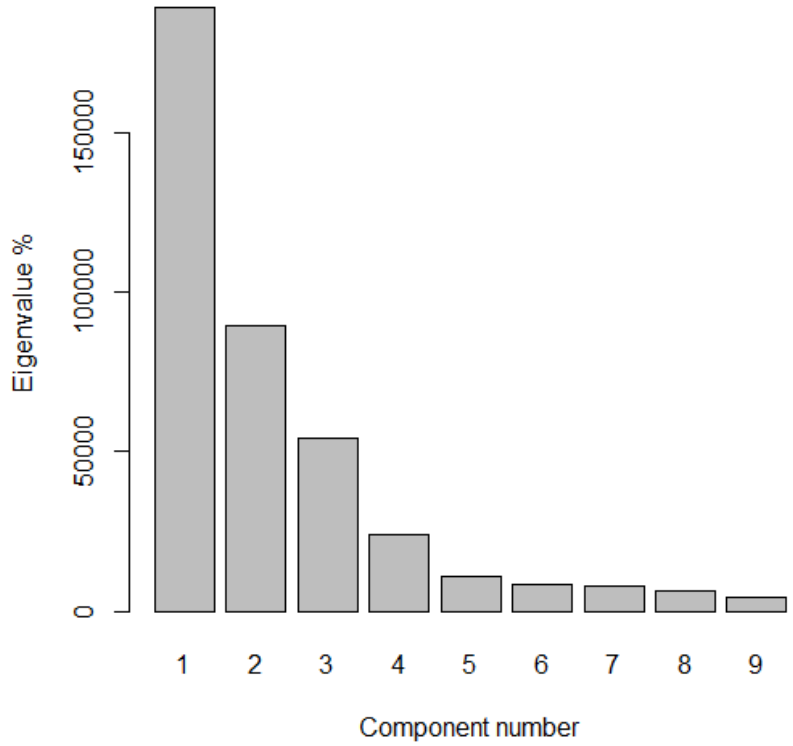


Figure 5.1 Scree plot of principal components. The curve of the scree plot suggests that using five PCs would be sufficient for the analysis, since the curve flattens after that point.

5.2.4 Magma gene analysis

MAGMA gene analysis was performed with the default test of mean SNP association, which uses sum of squared SNP Z-statistics as test statistic (de Leeuw et al. 2015). Analysis was restricted to 198 SNPs extracted from the PWS-cr region UKBB genotype data after quality control. The analysis was performed using the 1000 genomes European panel as reference data from CNCR CTGLab (https://ctg.cncr.nl/software/magma) and GWAS summary statistics to obtain SNP test results. Intelligence GWAS data were used for analysis of cognition (Savage et al. 2018). The Savage et al. (2018) study collated data from cohorts that included measures of cognition at least three aspects of performance, such as executive function, working memory and processing speed. These aspects of cognition were commonly measured by DST, SDST, verbal memory for words/stories, semantic/phonemic fluency, and trail-making tests such as TMTA and TMTB. The analysis itself was performed on a score calculated from the first unrotated principle component derived from the results of these tests, which was estimated to explain an average of 42% of variance in test performance. Summary statistics data from a MDD GWAS were taken from the Psychiatric Genomics Consortium for analysis of depression, which used a lifetime diagnosis of MDD determined through a clinical interviews/checklists, or medical record review (Wray et al. 2018). GWAS summary statistics of height, BMI and obesity were taken from the Giant consortium (Berndt et al. 2013; Yengo et al. 2018). SNP locations for annotation were extracted from each respective GWAS summary statistics dataset. Bonferroni correction was applied to correct for multiple testing, adjusting the p-value threshold for significance to 0.013.

5.2.5 Polygenic risk score analysis

Calculations of PRS of the whole genome and the PWS-cr region alone were performed with PRSice2, which uses p-value clumping and thresholding method and generates best fit PRS (Choi et al. 2020). Summary statistic data from the GWAS used in the MAGMA analysis were used as base data for PRS analysis; MDD GWAS data was used for the two depression outcomes analyses, the intelligence GWAS data was used for the seven cognition test analyses, the height GWAS data was used for the height analysis, BMI GWAS data was used for the BMI analysis, and the obesity GWAS data was used for the body fat percentage analysis. UKBB samples were excluded from all data sets. SNPs in the major histocompatibility complex (MHC) region on chromosome 6 were also excluded from the analysis due to the complex

linkage disequilibrium of the region. Ambiguous SNPs were excluded from all GWAS data.

Data were analysed with linear and logistic regressions using PRS scores from PWS-cr region as between-subject factors. Sex, age, age^2, PRS of the whole genome, and up to five whole genome variation principal components were added to the models as covariates. Bonferroni correction was applied to correct for multiple testing, adjusting the p-value threshold for significance to 0.004.

5.3 Results

5.3.1 Principal components analysis

The principal component analysis showed no correlation between the PWS-cr principal components and self-reported depression with an antidepressant prescription (SRDAP) or hospital discharge with depression (HDD). The analysis also showed no evidence of a link between the PWS-cr principal components and the results from the pairs matching test (PMT), the fluid intelligence (FIT), the digit span test (DST), the reaction time test (RTT), the symbol digit substitution test (SDST), the trail making test A (TMTA) and the trail making test (TMTB). The results from the analysis of height, BMI, and body fat percentage also showed no significant effect of the PWS-cr principal components on these traits after Bonferroni correction for multiple testing. Results from the statistical analysis can be found in Table 5.1.

Table 5.1 Results from statistical analysis of effect of PCs of genomic variation on traits of interest.

Trait	N	Test statistic	(pseudo) R^2	p-value
SRDAP	442 907	5.320	0.012	0.378
HDD	410 022	3.494	0.005	0.624
PMT	442 498	0.596	0.021	0.703
FIT	141 737	0.580	0.019	0.716
DST	45 585	0.712	0.026	0.615
RTT	439 929	0.937	0.113	0.046
SDST	107 330	1.992	0.026	0.583
TMTA	94 754	0.755	0.110	0.043
TMTB	94 752	1.821	0.116	0.105
Height	441 954	2.950	0.509	0.012
BMI	441 491	2.350	0.007	0.038
Body fat %	434 965	2.758	0.436	0.017

Threshold of significance = 0.004.

The results from the principal component analysis didn't demonstrate any significant effect of PWS-cr genetic variants represented by principal components on any of the examined phenotypes and traits. Notably, the whole genome PCs, which were used as covariates to correct for population stratification, had a significant ($p<0.004$) effect on all of the variables of cognition, but not on the variables of depression, height, BMI or body fat percentage, which would be expected to be affected by genetic heritage.

5.3.2 MAGMA gene analysis

The results from the MAGMA analysis did not demonstrate a link the SNPs in PWS-cr and MDD or intelligence. The results from the analysis of height also showed no significant link between variation in PWS-cr and the phenotype. Notably, the variation in *SNORD116* but not *IPW* was significantly correlated with BMI. Overall, the results of this analytical approach showed there might be a potential link between the SNPs in *SNORD116* and BMI, but none of the other examined phenotypes were linked to the variation of the PWS-cr. Results from the statistical analysis can be found in Table 5.2.

Table 5.2 Results from MAGMA analysis of traits of interest.

Trait	**n**	**Test statistic**	**p-value**
MDD and *SNORD116*	143 265	-0.354	0.711
MDD and *IPW*	143 265	-1.249	0.894
Intelligence and *SNORD116*	74 217	0.125	0.450
Intelligence and *IPW*	74 217	-0.354	0.638
Height and *SNORD116*	253 288	1.856	0.031
Height and *IPW*	253 288	1.492	0.068
BMI and *SNORD116*	339 224	2.460	0.007*
BMI and IPW	339 224	-1.694	0.045

Threshold of significance = 0.013. * indicates statistical significance

5.3.3 Polygenic risk score analysis

The results showed no correlation between the PRS of the PWS-cr region and self-reported depression with an antidepressant prescription or hospital discharge with depression, whereas both models showed a significant link between the traits of interest and the PRS of the whole genome ($p<0.001$).

In the examination of cognition, the results showed no link between the PWS-cr PRS and performance in the pairs matching test, the fluid intelligence test, the reaction time test, digit span test, trail-making test A, and trail-making test B, while all of these models showed a significant correlation between whole genome PRS and the traits of interest ($p<0.001$). Notably, the symbol digit substitution test was the only cognition test that showed evidence for a weak positive correlation between the PWS-cr PRS and test score,

The analysis also showed no evidence of a link between the PWS-cr PRS and height, BMI, and percentage body fat, while all three models showed a significant correlation between the phenotypes and genetic liability as represented by the whole genome PRS. Results from the statistical analysis can be found in Table 5.3.

Table 5.3 Results from PRS analysis of traits of interest.

Trait	n	Test statistic	(pseudo) R^2	p-value
SRDAP	363 336	-1.668	0.015	0.094
HDD	333 475	-1.501	0.008	0.134
PMT	363 114	1.305	0.022	0.194
FIT	112 619	-0.646	0.044	0.521
DST	36 635	1.803	0.038	0.230
RTT	360 940	1.188	0.114	0.067
SDST	86 104	3.158	0.210	0.001*
TMTA	75 856	-1.471	0.111	0.140
TMTB	75 854	-2.422	0.174	0.016
Height	362 655	1.319	0.623	0.187
BMI	362 281	-1.470	0.068	0.141
Body fat %	356 790	1.083	0.210	0.281

Threshold of significance = 0.004. * indicates statistical significance

Overall, the results of this analytical approach showed no evidence of an effect of the PWS-cr variants on depression, stature, obesity or cognition, with the exception of the symbol digit substitution test, which was positively correlated with the PRS generated from the PWS-cr variants, meaning that individuals with higher PRS performed better on the SDST. Notably, all of the examined traits were significantly linked to the whole genome PRS which demonstrates the genetic liability of the examined variables.

5.4 Discussion

The study in this chapter used three different analytical approaches to investigate whether genetic variation within the PWS-cr interval has an effect on depression and cognition. Overall, the results demonstrated no significant correlation between PWS-cr variants and the phenotypes of interest, with the exception of the symbol digit substitution test of cognition which was correlated with the PRS of the PWS-cr interval. These findings show no evidence that the genes in PWS-cr play a role in the increased prevalence of depression and MDD or in most of the cognitive deficits associated with PWS.

The symbol digit substitution test is commonly used to assess cognitive function in individuals with psychiatric and cognitive disorders as a measure of complex processing speed and associative learning (Walsh 1978; Jaeger 2018). Notably, this test has been linked to visual scanning and contour formation and to the rate of processing visual figures (Royer 1971; Glosser et al. 1977). There are no reports of the symbol digit substitution paradigm being used to test cognition in PWS. However, there are multiple studies that show that individuals with PWS have improved shape recognition abilities and significantly higher aptitude for jigsaw puzzle solving compared to their neurotypical peers (Roof et al. 2000; Dykens 2002; Rosner et al. 2004; Whittington et al. 2004). The results from the PRS analysis suggest that *SNORD116* and *IPW* in the PWS-cr interval might play a role in this cognitive phenotype of PWS.

None of the other variables of cognitive function or depression examined in this study showed evidence of being significantly linked to the variation in PWS-cr. As previously acknowledged in this chapter, cognition and depression are highly complex traits controlled by multiple loci, many of which might have small enough effects to be individually undetectable. It is therefore possible that the PWS-cr genes could have an effect on aspects of cognition and depression that would be missed by a target gene study like this one. Another weakness of candidate gene studies of complex traits is that the effect of a restricted set of SNPs on the phenotype of interested might be dependent on epistasis, while the target gene analysis is missing the data of the rest of the genome (Bosker et al. 2011).

Notably, the height and obesity traits that were examined as a comparison also didn't correlate significantly to the PRS and PCs of the PWS-cr. *SNORD116* and *IPW* have both been linked to weight and stature in studies of individuals carrying a deletion of the PWS-cr, and in studies of knock-out mouse models. Since the exact molecular

mechanisms through which both of these non-coding genes operate is unknown, it is difficult to estimate what kind of effect variation would have on their function. It's possible that the effect of PWS-cr variation on height and weight would be a subtler than the deletion of the region, and therefore it might not have been detected by the PRS and PCA analyses. Furthermore, the whole genome PCs, which were used to correct for population stratification in both PRS and PCA studies, also showed no significant link to these traits. Since the whole genome PCs represent genetic heritage, they would have been expected to have an effect on most if not all the examined traits, while they showed a significant link only on to the cognition traits. This suggests that the results from the PCA study might not be reliable, particularly for the depression, height and obesity phenotypes.

In contrast, the MAGMA analysis showed a link between SNPs in *SNORD116* and BMI. Since the MAGMA SNP-wise gene analysis does not use an input of phenotypes, the variables of interest were represented as four main categories through the GWAS datasets they were analysed with – depression, cognition, height, and BMI. The reduced number of studied variables lead to a much lower p-value threshold of significance after applying a Bonferroni correction. Therefore, it is possible that the testing of as many as 12 variables in combination with a very stringent multiple testing adjustment could have generated type II error in the PCA and PRS studies.

It also has to be acknowledged that the UK biobank sample is not an ideal representation of the population in the UK. The UKBB recruited participants over the age of 40 and is skewed towards higher socio-economic class and educational attainment (Fry et al. 2017), which are positively correlated with high cognition and lower instance of depression respectively (Deary et al. 2007; Strenze 2007). Furthermore, rare variants were excluded from the analysis, but it cannot be discarded that they could potentially have a causal role on the traits of interest.

In conclusion, the results of this study tentatively indicate a link between PWS-cr and shape-recognition aptitude which is common in individuals with PWS, but finds no evidence of the interval having a role in any of the other examined measures of cognition or depression. Due to the nature of target gene variation studies and the corrections for multiple testing, it is possible that some of these results are falsely negative, and further studies would be required to investigate whether PWS-cr has a role in the studied aspects of cognition and depression.

Chapter 6. General discussion

Prader-Willi syndrome is a neurodevelopmental disorder caused by mutations affecting the expression of an imprinted cluster of genes on chromosome 15. This disorder affects growth, weight, feeding behaviour, sleep, and sexual development, predominantly through loss of expression of the two non-coding RNAs on the PWS-cr interval (Butler et al. 1996; Sahoo et al. 2008; de Smith et al. 2009; Duker et al. 2010; Powell et al. 2013; Bieth et al. 2015; Fontana et al. 2017; Coulson et al. 2018; Polex-Wolf et al. 2018; Pace et al. 2020b).

Individuals with PWS also exhibit complex cognitive and psychiatric phenotypes, although the underlying molecular processes are still not well understood. The cognitive phenotypes of PWS are mostly attributed to the loss of expression of the PEGs of the PWS locus, but the exact genes contributing to these traits are unknown. Psychiatric illness, on the other hand, is considered to be mainly caused by the overexpression of the MEGs of the PWS locus (Sinnema et al. 2011; Yang et al. 2013; Noor et al. 2015; Isles et al. 2016), but there is evidence to suggest that the loss of expression of some of the PEGs affects the serotonergic system and contributes to psychiatric illness (Kishore and Stamm 2006b; Zanella et al. 2008; Doe et al. 2009; Mercer et al. 2009; Sinnema et al. 2011).

The research described in this thesis investigated whether the PWS-cr interval, containing two non-coding PWS PEGs, plays a role in phenotypes of relevance to cognition and psychiatric illness. I used three different approaches: characterising a range of relevant behaviours in the PWS-cr mouse for the first time, examining the transcriptomic profile of the neonatal brain of the PWS-cr mouse, and investigating whether the genetic variation within PWS-cr is linked to cognition or depression.

6.1 Behavioural characterisation of the PWS-cr mouse model

The behaviour of the PWS-cr mice had not been examined in aspects relevant to psychiatric illness and cognition, with the exception of a few studies including the EPM and OF tests (Ding et al. 2008; Zieba et al. 2015), and a single study that reported impaired object recognition and object location memory (Adhikari et al. 2019). In order to fill in this gap in the characterisation of the PWS-cr mouse and to investigate the role of PWS-cr, I conducted a series of tests examining behaviours relevant to anxiety (in the EPM and OF test), affective and psychotic illness (ASR and PPI tests) and attention and impulsivity (5-CSRTT). Crucially, a study in our lab had previously shown that most of these behaviours are affected in the PWS-IC mouse model, which carries a loss of expression of the PEGs of the PWS-locus, including

the PWS-cr interval. Conducting these studies allowed me to examine whether PWS-cr contributed to the behavioural phenotypes observed in the PWS-IC mouse model.

The results from the EPM and OF tests showed no evidence of anxiety, similar to what had been observed in the PWS-IC mouse model in the same conditions of the same lab (Relkovic et al. 2010). Previous studies of the PWS-cr mice in the OF and EPM have reported indications of anxiety in the EPM in particular (Ding et al. 2008; Zieba et al. 2015), which were not present in the results of this thesis. Notably, Zieba et al. (2015) reported reduced anxiety in the OF, while Ding et al. (2008) reported no indications of anxiety in the OF, as was observed in the behavioural study from Chapter 2 as well. These disparities between results from the same mouse model in the same tests could be due to differences in settings, which both EPM and OF are sensitive to (Lewejohann et al. 2006; Sousa et al. 2006). Studies have shown that EPM and OF have low replicability (Griebel et al. 2000; Milner and Crabbe 2008; Post et al. 2011). O'Leary et al. (2013) conducted analysis on EPM and OF data from 15 different strains of mice, to conclude that the traditionally measured variables at the two tests are more dependent on apparatus conditions and laboratory variables than on genetic differences. Overall, this suggests that the results from the EPM and OF might not be a good indicator on whether the genes in the PWS-cr interval play a role in anxiety and anxiety-related disorders in PWS.

A significant effect was observed in the ASR test, but not in the PPI. The PWS-cr mice exhibited a significantly reduced startle response, which has been reported in individuals with MDD and anhedonia, and as an effect of anxiolytic drugs (Riba et al. 2001; Commissaris et al. 2004; Kaviani et al. 2004). Interestingly, the PWS-IC exhibit a very different phenotype at the ASR and PPI tests, with a significantly increased startle response and an impairment of the pre-pulse inhibition, which is indicative of defective sensory-motor gating (Relkovic et al. 2010). Collectively, the phenotypes of the PWS-IC mouse model are linked to psychotic illness (Parwani et al. 2000; Akdag et al. 2003). This difference between the PWS-cr and PWS-IC models replicates the phenotypic differences between the psychiatric profiles of individuals with the delPWS genetic subtype who are prone to depression without psychotic illness versus those with the mUPD15, who are prone to psychotic illness which is sometimes accompanied by depression (Soni et al. 2008; Sinnema et al. 2011). Overall, the results from these tests suggested that PWS-cr does not contribute to behaviours relevant to psychosis, but it might play a role in the depressive phenotypes observed in individuals with PWS. In future studies, forced swimming or tail-hanging tests can be used to further investigate the role of PWS-cr for depression.

Some subtle differences of behaviour were also observed in the 5-CSRTT, where the PWS-cr mice exhibited a quicker response to the visual stimuli than their wild type littermates. This result suggests that the PWS-cr could have a slightly better attention than control. In humans, deletions of the PEGs of the PWS locus are associated with attentional deficits (Gross-Tsur et al. 2001; Wigren and Hansen 2005). The PWS-IC mouse model which carries deletion of the PWS PEGs, has also exhibited attentional deficits across almost all of the same variables in the same 5-CSRTT manipulations. It would be surprising to discover that in mice the loss of the PWS-cr interval is associated with the opposite phenotype. Although the PWS-cr region is conserved between humans and mice, there are some differences in the number of copies of *SNORD116* and exons of *IPW*, which could potentially be producing different outcomes between humans and mice. Furthermore, mouse models cannot fully recapitulate the neurodevelopmental bases of ADD and ADHD in humans, which could lead to different downstream outcomes of the PWS-cr deletion. However, that effect disappears a correction for multiple testing is applied and is likely that this outcome is the result of a type I error due to multiple testing.

The only other difference observed in the 5-CSRTT was in the progress through the manipulations. There was no difference in the number of training sessions taken to progress to baseline conditions, but once manipulations were introduced, the male PWS-cr mice took significantly higher number of sessions on average to reach stable baseline after each manipulation. This could be indicative of behavioural inflexibility, which is the inability to adapt to novel situations and can be linked to ASD and rodent models of ASD (Sanders et al. 2008; Geurts et al. 2009; Silverman et al. 2010). It is possible that the PWS-cr interval contributes to the inflexible behaviour and increased prevalence of ASD in individuals with PWS, but the 5-CSRTT is not adapted to study these kinds of phenotypes. In the future, perhaps a reversal learning task could be used on this mouse model in order to investigate whether the PWS-cr plays a role in these behaviours. It also has to be acknowledged that the PWS-cr males were the group with the lowest n, and analysis of sex-genotype interactions were underpowered, which could have influenced the results.

Overall, the PWS-cr mice did not exhibit phenotypic differences compared to control in most of the examined behaviours. The most notable difference observed was in the ASR, which showed a behaviour of relevance to MDD. The 5-CSRTT test also showed inflexible behaviour of relevance to ASD, but due to the limitations of these studies further investigation would be required to draw any conclusions. If this finding is solidified, the behavioural profiles of the PWS-IC and PWS-cr mouse models would

fits with Badcock and Crespi's (2008) theory of autism and psychotic illness being diametrically opposed disorders of the social brain linked to the maternal and paternal lineage respectively.

The PWS-IC mice, on the other hand, exhibited robust phenotypes of deficient attention at the 5-CSRTT and behaviours of relevance to psychotic illness at the ASR and PPI tests (Table 6.1). An informal comparison of the results from the PWS-cr and PWS-IC models suggests that the PWS-cr interval does not contribute to psychotic illness, and attentional deficits, but might play a role in the increased prevalence of depression observed in individuals with PWS. The PWS-IC mouse model has also exhibited impulsivity phenotypes of relevance to ADHD in the stop-signal reaction time task (Davies et al. 2019), which was not tested with the PWS-cr mouse due to time constrains, but it would be interesting to investigate in future studies.

It has to be acknowledged, that while the PWS-cr mice were bred on the same genetic background and tested on the same aperture as the PWS-IC mice, the two models were tested almost a decade apart by different people. Since most mouse behaviours are notoriously sensitive to the environment, different handling techniques or changes in the housing unit such as levels of noise could have an effect on these results.

Table 6.1 Behavioural differences between the PWS-cr and PWS-IC mice.

Behaviour	**PWS-cr**	**PWS-IC**
Anxiety (EPM)	↔	-
Anxiety (OF)	↔	↔
Relevant to affective and psychotic disorders (ASR)	↓	↑
Relevant to affective and psychotic disorders (PPI)	↔	↓
Attention (5-CSRTT)	↔	↓
Impulsivity (5-CSRTT)	↔	↔

6.2 Transcriptomic characterisation of the PWS-cr and PWS-IC mouse models

To examine the transcriptomic differences underlying the distinct behavioural profiles of these two mouse models, I performed an RNA-sequencing study of whole brain tissue and analysed differential gene expression and differential isoform usage. Although the behavioural studies were performed on adults, the psychiatric and cognitive disorders of relevance to PWS are of neurodevelopmental origin (Rapoport et al. 2005; Ansorge et al. 2007; Elia et al. 2010; Poelmans et al. 2011; Zhao et al. 2015), which justified the use of neonatal samples for the analysis.

The PWS-cr mouse model exhibited very minor differences in gene expression compared to control. One of the most notable findings from that model was the increase in expression levels of *Necdin* in the absence of PWS-cr. *Necdin* regulates neuronal outgrowth and differentiation and studies of its deletion in mice have shown that it has an effect on learning and memory (Muscatelli et al. 2000; Lee et al. 2005; Kuwajima et al. 2006). Since *Necdin* is expressed in the whole brain throughout adulthood (Uetsuki et al. 1996; Niinobe et al. 2000), in future studies it would be worth examining whether the expression of *Necdin* is affected in adult brain tissue, which might help understand the molecular relationship between the genes of PWS-cr and *Necdin.*

Another notable finding was that the PWS-cr mouse model exhibited increased usage of a truncated form of *Dyrk3*. In humans, *DYRK3* has been shown to work in synergy with *DYRK1A* to phosphorylate and activate Sirtuin1 deacetylase (*SIRT1*). Both *DYRK1A* and *SIRT1* have been robustly linked to intellectual disability (Guo et al. 2010; Courcet et al. 2012b; Ji et al. 2015; Luco et al. 2016; Dalal et al. 2019). Overall, this suggests that the PWS-cr might have a role in molecular processes that affect aspects of cognition.

The PWS-IC mice, on the other hand, exhibited more pronounced changes in gene expression, enriched for molecular and cellular processes related to oxygen transport, which is relevant to neurogenesis among other processes (Zhang et al. 2011; Nalivaeva et al. 2018). Among the differentially expressed genes was *Plp1*, which has a role in myelination and has been associated with mild intellectual disability, developmental delay and functional hemispheric asymmetries of relevance to language, face processing, emotional processing and visuo-spatial attention (Tkachev et al. 2003; Hausmann 2005; Inoue 2005; Qin et al. 2005; Lee et al. 2006; Ocklenburg et al. 2017; Ocklenburg et al. 2018).

The transcriptional study of PWS-IC also found increased usage of a truncated isoform of the *Gabrg3* GABA receptor, which has been linked to psychotic illnesses (Webb et al. 2008; Ahn et al. 2011; Taylor and Tso 2015). Due to the key role of the GABA receptors in the development of the nervous system, this differential isoform usage in the neonatal brain could be affecting a number of different neural processes and could lead to a different trajectory of neural development that results in psychiatric illness. The transcriptomic profile of the PWS-IC mouse exhibits links to a wide variety of neural processes of relevance to cognition and psychotic illness, which reflects the

highly increased prevalence of psychosis in individuals with the mUPD15 genetic subtype compared to those with the delPWS genetic subtype.

Notably, the differential expression of *Necdin* and *Dyrk3* from the PWS-cr mouse were not observed in the PWS-IC mouse. This could be a possible explanation for the distinct cognitive phenotypes between the delPWS and mUPD15 genetic subtypes, with the latter exhibiting significantly milder forms of learning disability than the former (Roof et al. 2000; Whittington et al. 2001).

The differentially expressed genes and isoforms from both mouse models were also analysed for enrichment of genetic variants relevant to psychosis and schizophrenia. The results showed no enrichment of variants relevant to either phenotype in the PWS-cr differentially expressed genes and isoforms, while the PWS-IC genes were enriched for common variants of psychosis, but not schizophrenia, which suggests distinct genetic liability of PWS psychosis from schizophrenia (Figure 6.1). The results from this study reflect the increased prevalence of psychosis associated with the mUPD15 genetic subtype of PWS, and show no evidence that the PWS-cr interval contributes to psychotic illness. Furthermore, these findings provide a genetic foundation for the clinical observations that the psychotic illness seen in PWS is distinct from schizophrenia (Soni et al. 2008), and should be treated as such. MRI scans of children with PWS have shown reduced white matter volume and differences in white matter structure similar to those observed in psychotic illness, including lower global fractional anisotropy and higher mean diffusivity (Lukoshe et al. 2013; Lukoshe et al. 2017a). In future studies, it would be worth designing a study to examine these structures in individuals with schizophrenia and individuals with PWS side by side.

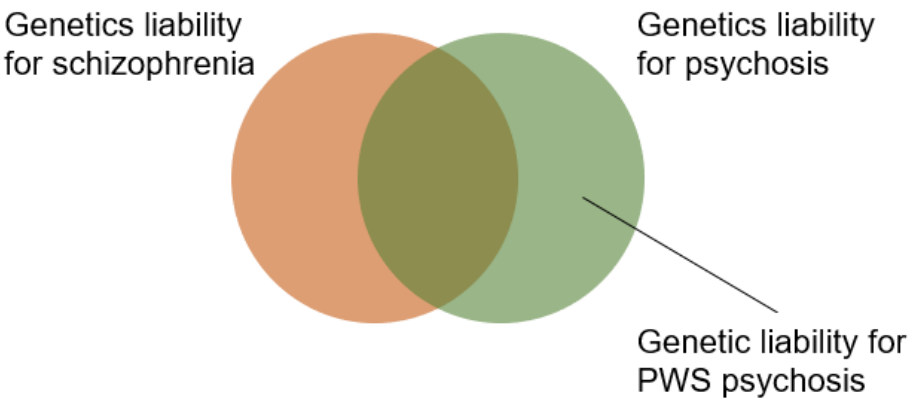


Figure 6.1 Model of genetic liability for psychosis in individuals with PWS. Gene set analysis of differentially expressed genes and differentially used isoforms in whole neonatal mouse brain showed enrichment of genes relevant to psychotic experiences, but not schizophrenia. Although schizophrenia and psychotic experiences have a shared genetic liability, they are ultimately distinct psychiatric conditions phenotypically and genetically. We propose that the genetic liability for psychosis in PWS is distinct from that of schizophrenia.

Notably, among the genes that carried common SNPs of psychotic experiences were the paternally expressed PWS genes *Magel2, Necdin, and Mkrn3,* which suggests a contribution of some of the PWS PEGs to these phenotypes. Repeating the behavioural experiments from this study on mouse models of PWS carrying deletions of these genes could help to further break down and unravel the genetics of PWS.

A limitation of this study was the use of whole brain tissue, which does not allow the localisation of these transcriptomic changes and results in some finer regional effects being diluted out and not showing up in this study. This also means that effects that were observed are in all likelihood quite robust. Furthermore, since the molecular role of the genes from the PWS-cr interval is not still fully understood, it was unclear in which tissues to expect downstream effects. The approach of using whole brain tissue allowed for a broader examination. Although *SNORD116* is expressed predominantly in the hypothalamus, it has been shown to affect DNA methylation and gene expression levels in the prefrontal cortex of adult mice (Coulson et al. 2018). In the future it would be interesting to run an RNA-sequencing study on neonatal tissue from the prefrontal cortex, which controls executive and cognitive function (Roberts et al. 1998; Funahashi and Andreau 2013; Paylor et al. 2018), especially in view of the results from the relationship between PWS-cr and *Necdin* and *Dyrk3.*

6.3 Investigation of the link between genetic variation in PWS-cr and depression and cognition

The study of the effects of genetic variation within PWS-cr discovered a correlation between visual figure recognition and PRS of the PWS-cr interval. Interestingly, the link to figure recognition could potentially be revealing of the genetic mechanisms behind the jigsaw solving aptitude that is observed in individuals with PWS (Roof et al. 2000; Dykens 2002; Rosner et al. 2004; Whittington et al. 2004). Based on these results it is possible to propose that one or both of the genes in the PWS-cr contribute to this unusual phenotype.

None of the other measures of cognition (episodic memory, simple processing speed, reasoning and problem solving, numeric working memory, and visual attention) were linked to variation within the PWS-cr. PWS-cr variants were also not significantly linked to the two examined variables of depression — self-reported depression with current antidepressant prescription and hospital discharge for depression. These results show no evidence of the genes within PWS-cr playing a role in depression or in most of the examined aspects of cognition, which could mean that PWS-cr does not contribute to these traits.

If that were the case, the increased prevalence of depression in individuals with PWS is either a product of some of the other gene dysregulations in the delPWS and mUPD15 genetic subtypes, or of the increased adversity caused by living with this syndrome which puts individuals with PWS at an increased risk of developing psychiatric illnesses (Chapman et al. 2004; Lesch 2004; Hettema et al. 2005; Matheson et al. 2013). The task of maintaining a healthy weight has been reported as a stressor for most individuals with PWS and the increased prevalence of ASD and OCD has also been shown to lead to frustration and anxiety when any changes in routine are introduced (Dykens et al. 1992; Wigren and Hansen 2003; Dykens 2004).

In light of this interpretation, the reduced startle response in the PWS-cr mice could also be interpreted differently. Recently, Wu et a. (2020) reported that a *Necdin*-null mouse exhibits disturbances in the noradrenergic system, which has been associated with depression, stress, sleep cycles in regulation by NPY neurons and, crucially, with a reduced startle response (von Coelln et al. 2004; Chandley et al. 2014; McCall et al. 2015; Singh et al. 2017). Ressler and Nemeroff (2000) proposed that the role of the noradrenergic system is not directly on depression, but rather on the individual neural systems in different regions of the brain, which contribute to different

symptomology of relevance to depression. Since the data from this thesis has shown that in the absence of PWS-cr the expression of *Necdin* is modulated, and since *SNORD116* is expressed in NPY neurons and regulates the expression of NPY, it is possible that the reduced startle is a result of noradrenergic disturbances in the PWS-cr mouse model that lead to disturbances in sleep and startle response, but not to some of the typical traits of depression relating as anhedonia and mood. This idea could be further tested in the future by repeating the behavioural experiments on the PWS-cr mice after administration of drugs that target the noradrenergic system.

It must be acknowledged that the lack of evidence of a link between PWS-cr variants and measures of depression and cognition does not prove that the genes of the critical interval do not play a role in these traits. One possible approach to further solidify these findings would be to use a Lasso regression analysis with all of the PWS-cr variants to further assess the effects of individual variants on the traits of interest.

Additionally, both cognition and depression are polygenic and any individual gene effects might be minor enough to get lost in a target study such as this one, especially since limiting the variants to a particular region excludes epistatic interactions which could be contributing to the traits of interest. The concurrent examination of the effect of PWS-cr variants on height and BMI, which have been robustly shown to be affected by the genes in the PWS-cr interval, also showed no link in two out of three analytical approaches. Notably, a link between PWS-cr variants and BMI was only observed in the approach that had a significantly lower number of variables and was thus less impacted by multiple testing correction. This suggests that some of the results observed in this study could be a result of type II error. Perhaps a less stringent correction method should be considered for future studies, or alternatively, a more targeted selection of phenotypes.

Furthermore, this study only examined common genetic variants, whereas it cannot be discounted that some rare genetic variants could have an impact on the traits of interest. In future studies it would be worth examining the effect of rare variants on cognition and depression.

Table 6.2 Summary of findings, limitations and future studies.

	Findings	Limitations	Future studies
Behavioural study of the PWS-cr mouse model	-- PWS-cr mouse model exhibited no evidence of behavioural phenotypes of relevance to stress and anxiety in the EPM and OF --No evidence of psychotic illness in the ASR and PPI --Reduced startle response in the ASR, which could be indicative of depressive states and MDD --No evidence of impulsivity in the 5-CSRTT --Weak evidence of improved attention compared to wild type in the 5-CSRTT as observed by less time taking for correct response at baseline conditions --Weak evidence of inflexible behaviour in the PWS-cr males at the 5-CSRTT as observed by increased number of sessions taken to reach stable baseline after each manipulation	--EPM and OF are easily influenced by environment --5-CSRTT is not ideally suited for impulsivity or behavioural flexibility --Examining multiple variables at the 5-CSRTT increased chance for false positive results --Low number of PWS-cr males in lead to reduced power of examinations of sex-genotype interactions	--Forced swimming and tail-hanging tests for further examination of depression in the PWS-cr mouse --Stop-signal reversal reaction task to further examine impulsivity in the PWS-cr mouse --Reversal learning task to further examine flexibility of behaviour in the PWS-cr mouse
Transcriptomic study of neonatal brain samples PWS-cr and PWS-IC mice	--Differential expression of *Necdin* and differential isoform usage of *Dyrk3* in the PWS-cr mice suggestive of a role of PWS-cr in cognition --Differential expression of *Plp1* and differential isoform usage of *Gabrg3* in the PWS-IC mice could be the reason for the psychotic-like behaviour observed previously in this mouse model and reflect the psychosis phenotype in individuals with the mUPD15 genetic subtype	--PWS-IC samples were only female, sex could confound the results --Using whole brain tissue does not allow us to localize where the transcriptomic changes are occurring and finer localized effects are likely lost	--Examine the transcriptomics of the adult brain of the PWS-cr mouse to see whether the effects observed here persist after development --Western blot of differentially expressed genes and isoforms from both models in order to confirm that these changes are translated into protein

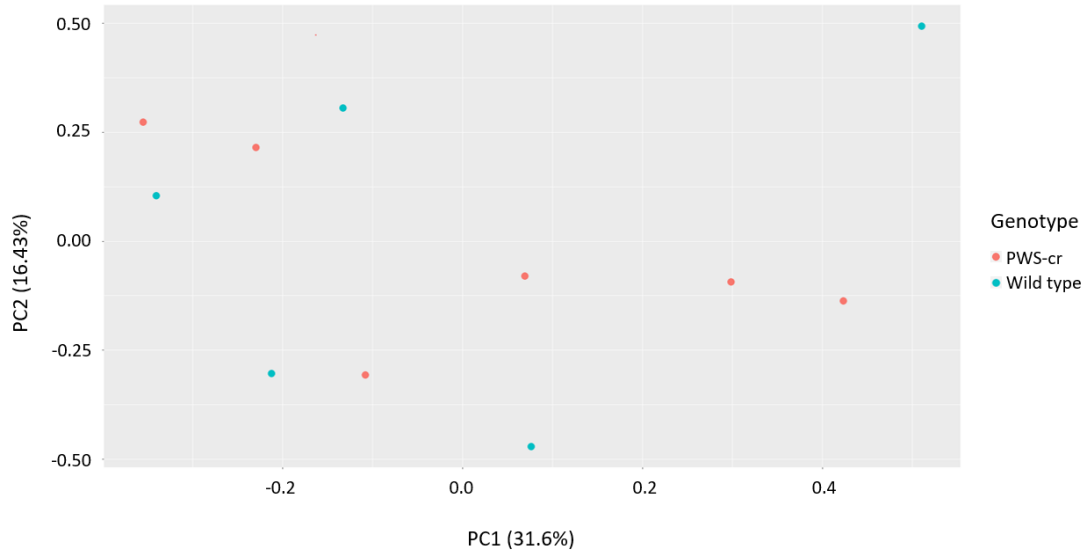
	Findings	Limitations	Future studies
Transcriptomic study	--Enrichment of genetic variants in relevance to psychosis but not schizophrenia in the PWS-IC brain samples and not in the PWS-cr solidify evidence that PWS-cr does not have a role in psychosis and suggest molecular pathways of psychosis in PWS distinct from schizophrenia		--RNA-sequencing of PWS-cr mouse prefrontal cortex tissue for more localised study of the role of PWS-cr --Neuroimaging of white matter microstructures in individuals with PWS vs individuals with schizophrenia
Genomic study	--Across three different approaches (PCA analysis, PRS analysis and MAGMA gene analysis), depression was not linked to common genetic variants of the PWS-cr interval --Most measured traits of cognition (episodic memory, simple processing speed, reasoning and problem solving, numeric working memory, and visual attention) also were not linked to variation within the PWS-cr --PRS analysis showed an effect of PWS-cr genetic variants on the results from a test that measures complex processing speed and shape recognition, which could potentially be linked to the improved jigsaw ability that is observed in individuals PWS -- Examination of height and BMI as a comparison showed no link to PWS-cr variation in PCA and PRS analyses, but BMI was correlated to PWS-cr variants in MAGMA gene analysis	--Significant effect of PWS-cr variation on weight and height in MAGMA but not in PRS and PCA analyses suggests that the multiple testing correction could have generated type II error --Candidate gene studies of genetic variation omit potential epistatic effects --UK biobank participants are over 40 and sample is skewed towards higher socio-economic class and educational attainment	--Use Lasso regression analysis with all of the PWS-cr variants (variable selection) to further assess the effects of individual variants on the traits of interest --Examination the effect of rare variants on the traits of cognition and depression

6.4 Conclusion

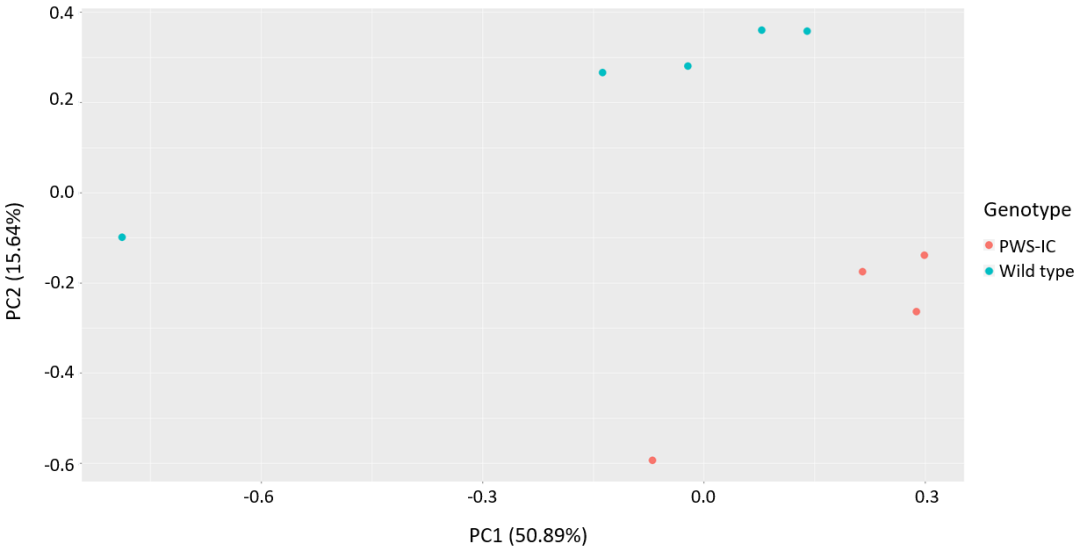
Collectively, the results from this thesis showed some evidence of the PWS-cr interval having a mild contribution towards the full psychiatric and cognitive profile of individuals with PWS. There was no evidence of PWS-cr having a role in psychotic illness or attentional deficits, but there is a tentative link to depression, behavioural inflexibility and an aspect of cognition. Further examination would be required to establish the role of the PWS-cr for these traits in hope that future findings would inform not only current understanding of the molecular bases of symptoms of PWS, but also of common psychiatric, behavioural and cognitive disorders such as MDD, ASD and ID.

Supplementary materials

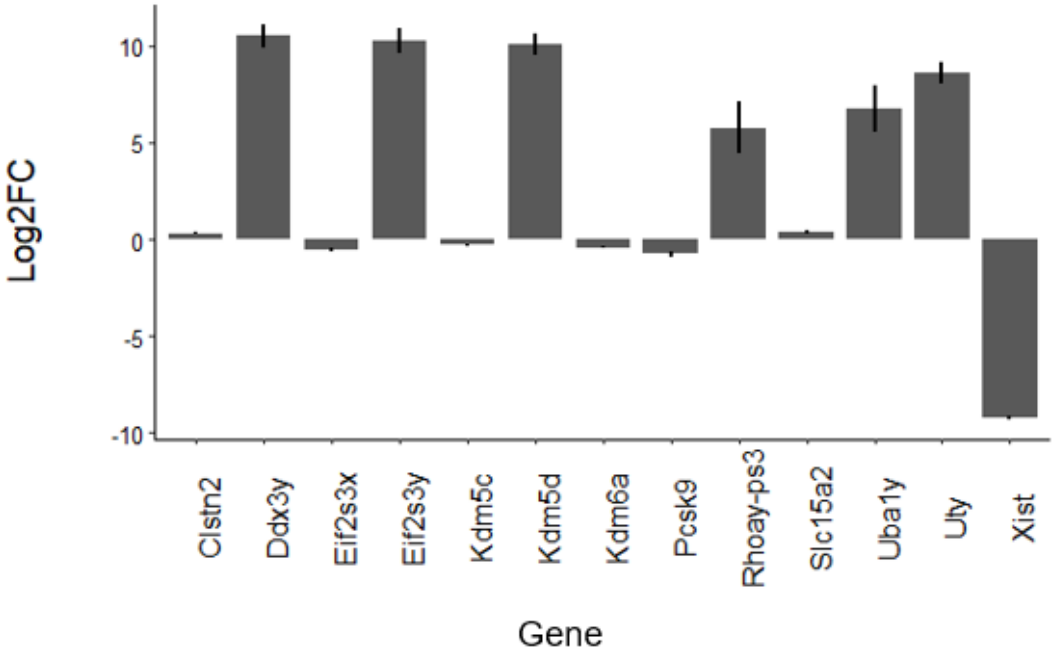
A)



B)



Supplementary figure 1. PCA of RNA samples from the PWS-cr mouse model (A) and from the PWS-IC mouse model (B). The PWS-cr samples did not cluster by group, while the PWS-IC samples mostly clustered by genotype, with the exception of one wild type and on heterozygous sample. This was not affected by sex, nor did the quality control or hierarchical clustering examinations show any problems with the quality of these samples.



Supplementary figure 2. Differentially expressed genes from the PWS-cr mouse model analysed by sex.

Supplementary table 1. Differential isoform usage from the PWS-cr mouse model analysed by sex.

Gene	Condition 1	Condition 2	Isoform 1	Isoform 2	q-value
Uty	female	male	0.951	0.592	5.57E-34
Uty	female	male	0.001	0.12	1.10E-30
Uty	female	male	0.004	0.107	1.87E-25
Kdm5d	female	male	0	0.165	1.97E-16
Itga8	female	male	0.034	0.24	3.06E-07
Rnf135	female	male	0.882	0.78	1.99E-05
Dnase2a	female	male	0.025	0.161	9.79E-05
Scarf1	female	male	0.146	0.013	0.00035
Kdm5d	female	male	0.444	0.286	0.000484
Zfp473	female	male	0.498	0.206	0.000508
Gm35315	female	male	0.977	0.656	0.000551
Rnf135	female	male	0.118	0.22	0.003871
Npff	female	male	0.853	0.958	0.005235
Fgd6	female	male	0.909	0.746	0.009199
Zfp473	female	male	0.233	0.627	0.009199
Efhc1	female	male	0.868	1	0.010769
Dna2	female	male	0.033	0.248	0.016979
Rassf6	female	male	0.121	0	0.026759
Dna2	female	male	0.571	0.419	0.047705

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