The rain to the wind said,
'You push and I'll pelt.'
They so smote the garden bed
That the flowers actually knelt,
And lay lodged--though not dead.
I know how the flowers felt.

-Robert Frost

University of Alberta

The *Magel2*-null mouse as a model of Prader-Willi Syndrome

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Medical Sciences – Medical Genetics

Fall 2012

Edmonton, Alberta

This thesis is dedicated to Gary Greenberg – who always knew that I would one day become a Doctor of some kind. I miss you.

Abstract

Prader-Willi Syndrome (PWS) is a neurodevelopmental disorder that results from the loss of several paternally expressed genes on chromosome 15q11-q13. People with PWS have an array of phenotypes including hypotonia and early failure to thrive, hypogonadism and infertility, growth hormone deficiency and short stature, childhood onset obesity and hyperphagia, and behavioural problems. Many of the features of PWS have been linked to hypothalamic dysfunction, but the contribution of individual genes lost in PWS and their role in hypothalamic development and function are poorly understood. In order to identify the contribution of individual PWS candidate genes to the PWS phenotype, transgenic mouse strains have been developed to examine consequences of gene loss in an animal model system. One of the genes lost in PWS, with very high expression in the hypothalamus is MAGEL2. Early studies of *Magel2*-null mice have suggested a role for *Magel2* in PWS-associated phenotypes, with circadian rhythm defects and reduced activity, as well as growth abnormalities including reduced weight gain prior to weaning, and increased weight gain and obesity as adults. In this thesis I describe subsequent investigations of the *Magel2*-null mouse, that confirm it as a key model for numerous aspects of PWS, including behavioural, reproductive, and energy balance abnormalities.

Magel2-null mice display subtle reductions in volume in discrete brain regions, neurochemical changes including reduced hypothalamic dopamine and serotonin, and behavioural abnormalities suggesting anxiety (Chapter 2). They have pubertal defects, reduced reproductive capacity and early infertility, and

reproductive changes including abnormal estrous cycles, reduced testosterone and olfactory impairments (Chapter 3). *Magel2*-null mice display leptin resistance prior to their development of obesity, and an absence of leptin-induced activation of pro-opimelanocortin neurons in the arcuate nucleus of the hypothalamus, a group of neurons linked to reductions in food intake and increased energy expenditure (Chapter 4). Taken together with previous findings, this work indicates a role for MAGEL2 in the pathophysiology of PWS. Future work targeting abnormal phenotypes in *Magel2*-null mice may suggest treatment possibilities for some features of PWS, including obesity.

Acknowledgements

First, I would like to thank my supervisor Dr. Rachel Wevrick for allowing me to join her laboratory and learn about Prader-Willi Syndrome while teaching me to be a scientist. For nearly 6 years, she has made herself available to me for guidance and assistance as I sometimes soared and sometimes struggled through my PhD. I would also like to thank her for her support as I ventured outside her laboratory and into the laboratory of my co-supervisor, Dr. William Colmers, to whom I owe great thanks for welcoming and supporting my work, and helping me learn about the neurobiology of energy balance regulation. His mentorship and support when I felt lost and confused are why this thesis became a reality.

Thank you to all the members of the Wevrick and Colmers laboratories, past and present, for your advice, assistance, and friendship. Alysa, Melissa, Sheldon, James, Dirk, Heika, Julia, Xiao, Erin, and Jason – you truly made me enjoy my time in grad school. I am especially thankful for Jocelyn in the Wevrick laboratory who has not only been an amazing teacher, but an open ear for my rants about both science, and the drama I often perceive in life. In the Colmers laboratory, Dr. Christopher Price showed infinite patience teaching me about electrophysiology, while often engaging me in spirited conversation about current events. He kept me grounded while providing encouragement and support, and for this I am particularly thankful.

Without the friendship of Lindsey in Medical Genetics and Jen in Pharmacology, I would have felt lost and alone here in Edmonton. Thank you so much for helping me build a home here and for making me feel loved and cherished. Your support means more to me than I can express. I love you.

Finally, I owe a mention to Manytoes, my wonderful cat who came to Edmonton with me and did nothing but give me love and affection no matter how stressed or busy I have been. And my other cat Daisy, who always makes me smile, deserves thanks for stopping me from working when it was needed by laying down on the keyboard or attacking me from behind. In the words of Jean Cocteau, *I love cats because I enjoy my home; and little by little, they become its visible soul.*

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

aCSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
AgRP	agouti related peptide
AHA	anterior hypothalamic area
ARC	arcuate nucleus
AS	Angelman Syndrome
AVP	arginine vasopressin
BBS	Bardet-Biedl syndrome
CART	cocaine and amphetamine regulated transcript
CNS	central nervous system
CRF	corticotropin releasing factor
DA	dopamine
DI	discrimination index
DIO	diet-induced obesity
DMN	dorsomedial nucleus
DOPAC	dihydroxyphenylacetic acid
E	embryonic day
EGFP	enhanced green fluorescent protein
FDR	false discovery rate
FSH	follicle stimulating hormone
GABA	gamma-aminobutyric acid
GAL	galanin
GALP	galanin-like peptide

GFP	green fluorescent protein
GH	growth hormone
GHRH	growth hormone releasing hormone
GnRH	gonadotropin releasing hormone
hCG	human chorionic gonadotropin
HPG	hypothalamic-pituitary-gonadal
HPLC	high-performance liquid chromatography
HVA	homovanillic acid
IC	imprinting centre
IHC	immunohistochemistry
ip	intraperitoneal
LepR	leptin receptor
LH	lateral hypothalamus
LH	leutenizing hormone
LHRH	leutenizing hormone releasing hormone
lPOA	lateral preoptic area
MAGE	melanoma antigen
MCH	melanin-concentrating hormone
ME	median eminence
MHD	MAGE homology domain
miRNA	micro RNA
MN	mammillary nucleus
mPOA	medial preoptic area
mRNA	messenger RNA
MRI	magnetic resonance imaging

MT-II	melanotan II
NA	noradrenaline
NPY	neuropeptide Y
n.s.	not significant
OC	optic chiasm
OCD	obsessive compulsive disorder
ORX	orexin
OT	oxytocin
P	postnatal day
PBS	phosphate buffered saline
PHN	posterior hypothalamic nucleus
PNS	peripheral nervous system
POA	preoptic area
POMC	pro-opiomelanocortin
PVN	paraventricular nucleus
PWS	Prader-Willi Syndrome
RMP	resting membrane potential
RNA	ribonucleic acid
SCN	suprachiasmatic nucleus
SEM	standard error of the mean
Sim1	single-minded 1
snoRNA	small nucleolar RNA
SON	supraoptic nucleus
SSRI	selective serotonin reuptake inhibitor
TH	tyrosine hydroxylase

TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone
UPD	uniparental disomy
VMN	ventromedial nucleus
WT	wild-type
5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin
α-MSH	α-melanocyte stimulating hormone

Chapter 1. Introduction

1.1 Prader-Willi Syndrome (PWS)

1.1.1 Clinical Findings in PWS

In 1887, Dr. J. Landon Down described an adolescent female with obesity, hypogonadism, short stature, and cognitive impairment (Down., 1887), attributed as the first clinical description of Prader-Willi Syndrome (PWS), a neurodevelopmental disorder identified in a group of children with similar features combined with hypotonia in the mid-1950s (Prader *et al.*, 1956). The prevalence of PWS has been estimated between 1/10,000 and 1/30,000, and occurs equally in both males and females among all races (Cassidy *et al.*, 2012). In 1981, it was determined that deletions on chromosome 15 are responsible for causing PWS (Ledbetter *et al.*, 1981). Genetic mechanisms of PWS will be described later in this chapter.

Major clinical features of PWS include hypotonia and feeding difficulties during infancy, excessive weight gain during early childhood, hypogonadism, hyperphagia, developmental delay, and characteristic facial features. Minor features in PWS include decreased fetal movements, behavioural problems, sleep apnea, short stature and growth hormone (GH) deficiency, small hands and feet, hypopigmentation, thick, viscous saliva, esotropia or myopia, speech articulation defects, and skin picking. A number of supportive findings have also been described, including high pain threshold, decreased vomiting, osteoporosis, kyphosis or scoliosis, early adrenarche, adrenal insufficiency, thermoregulation defects, unusual skill with jigsaw puzzles, and normal neuromuscular studies

(Holm *et al.*, 1993). These features have been used to create a clinical diagnostic scale for PWS, which is shown in Table 1-1.

Though the diagnostic scale developed by Holm *et al.* (1993, see Table 1-1) has been demonstrated accurate in the diagnosis of PWS (Gunay-Aygun *et al.*, 2001), diagnosis of PWS now relies on genetic testing. Accordingly, features of PWS are now used to prompt genetic testing to confirm the diagnosis. Because these features differ by age group, specific criteria have been outlined for different ages as shown in Table 1.2. Specific genetics of PWS are discussed below.

Table 1.1 Diagnostic criteria for Prader-Willi Syndrome

	Major Criteria (1 point each)
1	Neonatal/infantile hypotonia and poor suck
2	Feeding problems and failure to thrive during infancy
3	Weight gain between age 1-6; obesity in the absence of intervention
4	Characteristic facial features (including low set ears, almond shaped and down slanted eyes, narrow face, small mouth with thin upper lip)
5	Hypogonadism
6	Developmental Delay
7	Hyperphagia/food foraging/obsession with food

	Minor Criteria (1/2 point each)
1	Decreased fetal movement
2	Typical Behavioural problems (such as temper tantrums, violent outbursts, oppositional, manipulative, posessive, stealing, lying)
3	Sleep disturbance of sleep apnea
4	Short stature for family by age 15
5	Hypopigmentation for the family
6	Small hands and feet for height
7	Narrow hands with straight ulnar border
8	Eye abnormalities (esotropia, myopia)
9	Thick, viscous saliva
10	Speech articulation defects
11	Skin Picking

	Supportive criteria (no points but strengthen likelihood of diagnosis)
1	Increased pain threshold
2	Decreased Vomiting
3	Scoliosis or Kyphosis
4	Early adrenarche
5	Osteoporosis
6	Thermoregulation defects
7	Unusual skill with jigsaw puzzles
8	Normal neuromuscular studies

Clinical diagnosis requires five points (at least four of them major) at age < 3 years; 8 points (at least 5 of them major) at age > 3 years. Adapted from (Holm et al., 1993)

Table 1.2 Recommended Criteria to Prompt DNA Testing for PWS.

Age at Assessment	Features Prompting DNA Testing
0-2 Years	Hypotonia with poor suck
2-6 Years	Hypotonia with history of poor suck Developmental delay Growth failure and accelerated weight gain
6-12 Years	History of hypotonia with poor suck (hypotonia often persists) Developmental delay Hyperphagia, central obesity if diet uncontrolled
13 Years - Adult	Cognitive Impairment (usually mild intellectual disability) Hyperphagia, central obesity if diet uncontrolled Hypothalamic hypogonadism Characteristic behaviour problems

Adapted from (Gunay-Aygun et al., 2001; Cassidy et al., 2012)

1.1.2 Genetics of PWS

PWS is caused by the loss of expression of genes on the paternally derived chromosome 15q11-q13 (Figure 1.1) (Driscoll *et al.*, 1992, Nicholls and Knepper, 2001). This region of chromosome 15 is differentially imprinted during gametogenesis, resulting in maternal or paternal specific gene expression, rather than bi-allelic expression which is seen in non-imprinted genes (Driscoll *et al.*, 1992). The majority of PWS cases (~70%) result from a spontaneous microdeletion of the paternally inherited 15q11-q13 region (Ledbetter *et al.*, 1981, Butler and Palmer, 1983, Knoll *et al.*, 1989), another 20-25% of cases result from maternal uniparental disomy (UPD) for chromosome 15 (Nicholls *et al.*, 1989, Mascari *et al.*, 1992), and the remaining 5-10% of cases result from imprinting defects or chromosome 15 translocations that lead to altered methylation and/or disruption of the paternal 15q11-q13 region (Sutcliffe *et al.*, 1994, Buiting *et al.*, 1995, Grugni *et al.*, 2008). Loss of the maternally expressed 15q11-q13 region, specifically the UBE3A gene, results in a different disorder, Angelman Syndrome (AS) (Driscoll *et al.*, 1992, Kishino *et al.*, 1997, Fang *et al.*, 1999).

PWS is a contiguous gene disorder, as it has been shown that the complete phenotype results from loss of expression of several genes. In the PWS paternal-only expressed region, there are five polypeptide-coding genes (*MKRN3*, *NDN*, *MAGEL2*, and the bicistronic *SNURF-SNRPN*); *C15orf2* (a gene biallelically expressed in testis but only expressed from the paternal allele in brain); several clusters of C/D box small nucleolar RNA genes (snoRNAs)(including *SNORD115/HBII-52* and *SNORD116/HBII-85*);

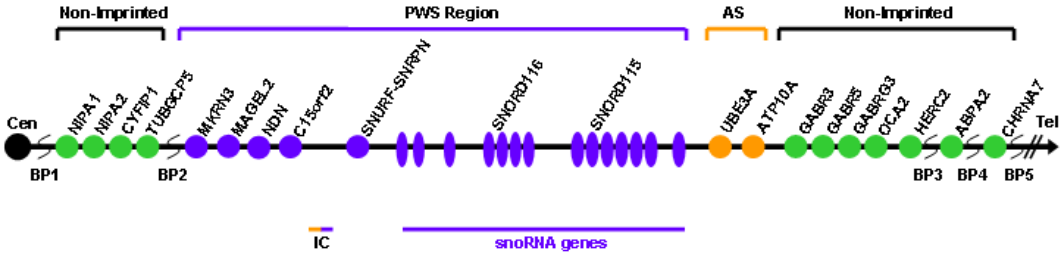


Figure 1.1 Organization of the Prader-Willi/Angelman Syndrome region on chromosome 15q11-q13.

Genes shown in blue are maternally imprinted/paternally expressed and their absence leads to PWS. Genes shown in orange are paternally imprinted/maternally expressed and their absence leads to AS. Genes shown in green are not imprinted, and biallelically expressed. BP refers to breakpoints, which are regions of repeated DNA segments with high rates of recombination. IC, imprinting centre; Cen, centromere; Tel, telomere. *Adapted from (Cassidy et al., 2012).*

and several antisense transcripts (Figure 1.1) (Cassidy *et al.*, 2012). The full PWS region also includes a number of non-imprinted genes with biparental expression: *NIPA1, NIPA2, CYF1P1* and *GCP5* between the two proximal breakpoints (Chai *et al.*, 2003), and a cluster of three GABA receptor genes, *OCA2*, and *HERC2* near the distal breakpoint.

The *NDN* and *MAGEL2* genes both produce proteins that belong to the melanoma antigen (MAGE) protein family. The MAGE family of proteins is characterized by the MAGE homology domain (MHD), a region involved in protein-protein interactions (Barker and Salehi, 2002). The physiological function of MAGE proteins remains largely unknown, though evidence points to important roles in cell cycle progression and apoptosis (Sang *et al.*, 2011). MAGE protein interactions through the MHD can act to prevent activation of the interacting protein, and/or prevent their binding to different proteins involved in a number of cellular processes. For example, one MAGE protein, MAGE-D1 (also known as NRAGE or Dlxin-1), binds the p75 neurotrophin receptor (P75NTR) and prevents its association with the tyrosine kinase receptor TrkA, leading to inhibition of cell cycle progression, and facilitation of p75NTR mediated apoptosis (Salehi *et al.*, 2000). MAGE-D1 is able to downregulate TrkA expression, and can inhibit nerve growth factor induced neuronal differentiation by regulating TrkA signaling (Feng *et al.*, 2010). MAGE-D1 knockout mice have been used to show that interaction of MAGE-D1 with nuclear receptors affects circadian rhythm (Wang *et al.*, 2010), and interactions with the serotonin transporter regulate depression-like behaviour (Mouri *et al.*, 2012). Thus, the MAGE protein family has the ability to regulate

numerous signaling pathways, leading to changes in various physiologic functions such as cell cycle regulation and behaviour.

NDN is a small, intronless gene which is widely expressed from early development through adulthood, with high levels of expression in the central and peripheral nervous systems (CNS, PNS) (Lee *et al.*, 2000, Muscatelli *et al.*, 2000, Niinobe *et al.*, 2000). Necdin has been shown to play numerous cellular roles, and is especially important during development. Necdin promotes the differentiation of neurons (Maruyama *et al.*, 1991, Kuwajima *et al.*, 2006, Takazaki *et al.*, 2002), skeletal (Kuwajima *et al.*, 2004, Deponti *et al.*, 2007, Bush and Wevrick, 2008), and smooth muscle (Brunelli *et al.*, 2004); plays a role in the differentiation and proliferation of adipocytes (Fujiwara *et al.*, 2012, Bush and Wevrick, 2012); is involved in the polarization of the cytoskeleton during development (Bush and Wevrick, 2010); promotes cell cycle exit (Hayashi *et al.*, 1995, Taniura *et al.*, 2005, Kuwako *et al.*, 2004); and inhibits apoptosis (Deponti *et al.*, 2007, Taniura *et al.*, 1999, Andrieu *et al.*, 2006, Aebischer *et al.*, 2011). Like *NDN*, *MAGEL2* is a small, intronless gene possessing a MHD. The expression of *Magel2* in mice almost exclusively found in the CNS, with especially high levels found in both developing and adult hypothalamus (Lee *et al.*, 2000, Boccaccio *et al.*, 1999). Peak levels of expression are seen in the mouse brain between embryonic day (E) 15-E17, which corresponds to the peak of neurogenesis. Also, like necdin, Magel2 has been shown to interact with *FEZ1* (fasciculation and elongation protein zeta 1), which further implies that Magel2 is involved in neuronal development (Lee *et al.*, 2005). Despite these findings specific cellular roles for the MAGEL2 protein

remains unknown, though its physiological roles are emerging through studies of *Magel2*-null mice, which are the major topic of this thesis.

The *SNURF-SNRPN* gene is bicistronic and encodes two different proteins: SmN, a spliceosomal protein involved in mRNA processing, and *SNURF*, a polypeptide of unknown function (Glenn *et al.*, 1996, Gray *et al.*, 1999). Proximally, the *SNURF-SNRPN* locus overlaps with the PWS imprinting centre (IC). *SNURF-SNRPN* serves as a host for the six snoRNA genes located telomerically, and the *UBE3A* antisense transcript arises from transcription of *SNURF-SNRPN*, and is believed to repress the paternal *UBE3A* gene (Runte *et al.*, 2001, Chamberlain and Brannan, 2001).

Numerous snoRNAs are present in the PWS region, most with only a single copy, though *SNORD115* (previously named *HBII-52*) and *SNORD116* (previously named *HBII-85*) are present in 42 and 29 copies respectively. snoRNAs are believed to play roles in RNA processing, including regulating alternative splicing and serving as precursors for microRNAs (miRNA), which play a role in RNA silencing (Holley and Topkara, 2011). It is thought that a given snoRNA might have multiple targets, but at this time in humans, only one target for one PWS snoRNA gene (*SNORD115)* is known: the serotonin 2C receptor (Kishore and Stamm, 2006, Doe *et al.*, 2009). In mice, *Snord115* has also been shown to regulate alternative splicing for 5 additional genes: *DPM2*, *TAF1*, *RALGPS1*, *PBRM1* and *CRHR1 (Kishore et al., 2010).*

Little is known about the remaining PWS candidate genes. *MKRN3* (also known as *ZNF127*) encodes a RING zinc finger protein that is widely expressed

but the function of this protein is unknown (Jong *et al.*, 1999a, Jong *et al.*, 1999b). *C15ORF2* appears to code for a protein expressed in testis and brain, but has no mouse ortholog and presently has no known function (Wawrzik *et al.*, 2010).

Recently, three patients with microdeletions encompassing only the *SNORD116* gene cluster have been described with multiple features of PWS including neonatal hypotonia, hyperphagia and obesity, hypogonadism, developmental delay, and speech and behavioural problems (Sahoo *et al.*, 2008, de Smith *et al.*, 2009, Duker *et al.*, 2010). These patients suggest that the majority of the PWS phenotype can be attributed to loss of this snoRNA cluster. However, these patients do not recapitulate the full PWS phenotype, with normal height, and no or atypical characteristic facial features. Additionally, a female patient with an atypical chromosome 15 deletion encompassing *MKRN3*, *MAGEL2*, and *NDN* also displays obesity and developmental delay (Kanber *et al.*, 2009), suggesting that though *SNORD116* may be especially important in PWS, the contribution of other genes cannot be ruled out.

1.1.4 Brain Regions Implicated in PWS

Many of the clinical features of PWS, including hypogonadism, short stature, hyperphagia, sleep disorders, thermoregulation defects, and central adrenal insufficiency are a result of hypothalamic dysfunction. No structural abnormalities have been observed in the hypothalamus of people with PWS, and though a reduction in the number of oxytocin (OT) neurons has been observed in post-mortem PWS brain samples (Swaab *et al.*, 1995), normal numbers of vasopressin neurons (Swaab *et al.*, 1995) and growth hormone releasing hormone

(GHRH) neurons (Goldstone *et al.*, 2003) have been shown. It also appears that the levels of the important hypothalamic neuropeptides neuropeptide Y (NPY) and agouti-related peptide (AgRP), both involved in increasing appetite, are normal in PWS subjects (Goldstone *et al.*, 2002), further suggesting that the hypothalamic defect in PWS is functional rather than structural. The functions of the hypothalamus are discussed later in this chapter.

Neuroanatomic studies have revealed a number of defects in people with PWS, though most of these defects are not fully penetrant and the implications of these findings remain unknown. All people with PWS appear to have enlarged ventricles (Hashimoto *et al.*, 1998, Miller *et al.*, 2007b). Other notable defects include small brainstems (Hashimoto *et al.*, 1998), Sylvian fissure abnormalities (Leonard *et al.*, 1993, Miller *et al.*, 2007a), incomplete insular closure (Miller *et al.*, 2007b, Miller *et al.*, 2007a), and decreased volume of tissue in the parietal-occipital lobe (Miller *et al.*, 2007b). Various abnormalities of the pituitary gland have also been described, including hypoplastic or globular pituitary glands (Miller *et al.*, 2008), and reduced pituitary height (Iughetti *et al.*, 2008).

1.1.5 Behavioural Phenotypes in PWS

People with PWS have a number of behavioural issues beginning in childhood that greatly impact quality of life. When creating the consensus diagnostic scale for PWS, Holm *et al.* (1993) outlined typical PWS behavioural issues including temper tantrums, violent outbursts, obsessive and/or compulsive behaviour, stealing, lying, and tendencies to be argumentative, oppositional, rigid, manipulative, possessive, and stubborn. Many of these behavioural problems

including temper tantrums, manipulation, and stealing are often increased in association with food seeking and nutritional control.

Obsessions and repetitive compulsive behaviours have emerged as central behavioural features in PWS (Dykens *et al.*, 1996, State *et al.*, 1999, Wigren and Hansen, 2003, Clarke *et al.*, 2002). In many people with PWS, these behaviours are time consuming, distressing, and lead to adaptive impairment, suggesting full blown obsessive compulsive disorder (OCD) (Dykens *et al.*, 1996). Some of the behavioural symptoms in PWS are suggestive of autism, and the comorbidity of autism spectrum disorders in PWS has been estimated to be between 19-37% (Veltman *et al.*, 2004, Milner *et al.*, 2005, Descheemaeker *et al.*, 2006). Psychiatric illnesses such as bi-polar disorder and psychotic episodes have also been described in people with PWS, and are far more frequent in those with maternal UPD or IC defects, suggesting gene dosage effects contributes to psychiatric illness in PWS (Soni *et al.*, 2007, Soni *et al.*, 2008, Sinnema *et al.*, 2011). Management of behavioural difficulties in PWS includes setting clear, consistent routines, occupational and speech language therapies, and the use of various pharmacological agents such as mood stabilizers, selective serotonin reuptake inhibitors (SSRIs), and antipsychotics (Dykens and Shah, 2003). However, individual cases of PWS vary greatly, demanding that behavioural therapy is designed on a patient to patient basis.

1.1.6 Reproductive Function in PWS

Hypogonadism is a major feature of PWS, with cryptorchidism, small testes, and scrotal hypoplasia in males and hypoplasia of the labia minora and/or

clitoris in females (Crino *et al.*, 2003). Although the hypogonadism in PWS has long been considered hypothalamic in nature, with low gonadotropins and subsequent low gonadal sex hormones, recent studies indicate primary gonadal deficiencies in both males in females as contributing factors (Hirsch *et al.*, 2009, Eldar-Geva *et al.*, 2009, Eldar-Geva *et al.*, 2010). Hypogonadism causes delayed, incomplete, and/or disordered pubertal development in both males and females with PWS, though normal or even precocious adrenarche is described (Crino *et al.*, 2003, Hirsch *et al.*, 2009, Eldar-Geva *et al.*, 2010). Females with PWS display primary amenorrhea or oligomenorrhea and though there have been instances of successful pregnancies in PWS females (Akefeldt *et al.*, 1999, Schulze *et al.*, 2001), male fertility has never been reported. Treatment of hypogonadism in PWS males with testosterone remains controversial, as reports suggest worsening of the PWS behavioural phenotype (Crino *et al.*, 2003). Recently, human chorionic gonadotropin (hCG) has been used to address hypogonadism in a cohort of male PWS patients, showing beneficial effects on virilization and normalized muscle mass (Eiholzer *et al.*, 2007).

1.1.7 Nutritional Phases and Obesity in PWS

Traditionally PWS has been described as having two distinct nutritional phases: hypotonia and early failure to thrive followed by hyperphagia leading to obesity (Gunay-Aygun *et al.*, 2001, Goldstone., 2004). Recently though, the progression between nutritional phases has been shown to be much more complex, with seven different nutritional stages described (Table 1.3) (Miller *et al.*, 2011). Phase 0 occurs *in utero* with decreased fetal movements, and reduced

birth weight and length in PWS compared to their unaffected siblings. In Phase 1, the infant is hypotonic with sub-phase 1a characterized by feeding difficulties often leading to failure to thrive. Phase 1a (birth to ~2 years of age) continues into sub-phase 1b, when the infant grows steadily with weight increasing normally. Phase 2 (~2-8 years of age) is where weight gain becomes apparent, with phase 2a showing increased weight gain without change in appetite or caloric intake. In phase 2b (median onset 4.5 years), increased interest in food first becomes apparent, with continued weight gain. Phase 3 is when true hyperphagia becomes apparent, with food seeking behaviours and lack of satiety. Some adults with PWS progress to Phase 4, which is defined as when an individual previously in Phase 3 no longer has an insatiable appetite and can feel full (Miller *et al.*, 2011). The hyperphagia characterized in Phase 3 is believed to be a result of hypothalamic dysfunction causing lack of satiety (Goldstone., 2004, Miller *et al.*, 2011).

Table 1.3 Nutritional Phases in Prader-Willi Syndrome

Phase	Median ages	Clinical characteristics
0	Before birth	Reduced fetal movements, low birth weight
1a	0-9 months	Hypotonia, feeding difficulties, reduced appetite
1b	9-25 months	Improved appetite and feeding, appropriate growth
2a	2.1-4.5 years	Weight gain without increase in appetite or caloric intake
2b	4.5-8 years	Continued weight gain, increased caloric intake and appetite, food preoccupation
3	8 years- adult	Hyperphagia, lack of satiety
4	adulthood	Appetite is no longer insatiable

Adapted from Miller et al., 2011

Obesity in PWS is primarily central, with reduced visceral fat levels for the degree of obesity (Goldstone *et al.*, 2001). Obesity in PWS is a result of hyperphagia and food seeking behaviours combined with decreased caloric requirements resulting from reduced activity levels and decreased lean mass compared with unaffected obese individuals (Butler *et al.*, 2007). People with PWS have significantly elevated levels of ghrelin (Cummings *et al.*, 2002, DelParigi *et al.*, 2002, Haqq *et al.*, 2003a, Goldstone *et al.*, 2004). Ghrelin is an appetite stimulating hormone produced mainly by the stomach that acts in the hypothalamus. While it is tempting to consider hyperghrelinemia as a cause for hyperphagia in PWS, it has been shown that increases in ghrelin levels precede the development of hyperphagia in PWS (Erdie-Lalena *et al.*, 2006, Feigerlova *et al.*, 2008), and pharmacological treatments that reduce ghrelin levels do not affect weight, appetite, or eating behaviours in PWS (Haqq *et al.*, 2003b, Tan *et al.*, 2004, De Waele *et al.*, 2008). The role of elevated ghrelin in PWS remains unclear, and no other hormonal abnormalities related to hyperphagia have been identified in PWS. Still, obesity and its consequent comorbidities remains the largest cause of morbidity and mortality in PWS and is a critical area of current investigation.

1.1.8 Mouse Models of PWS

Because numerous genes are inactivated in PWS, it is difficult if not impossible to determine how each gene contributes to the PWS phenotype. A region of conserved synteny for the PWS/AS critical deletion region was found on mouse chromosome 7C (Chaillet *et al.*, 1991), facilitating the creation of mouse

models of PWS in which all or only single PWS candidate genes have been eliminated. Though species differences between mice and humans confounds the interpretations of findings in these mouse models, similarities between PWS and mouse models of PWS highlight a role for these models in understanding the function of PWS candidate genes in a mammalian system.

PWS models with disruption of the entire PWS region

The first PWS mouse model used the *IS1CtX* autosome mouse, which has a translocation of a portion of mouse chromosome 7 containing the PWS/AS region to the X chromosome. Females carrying an unbalanced translocation with two copies of chromosome 7 plus the portion translocated to the X chromosome were mated with males carrying a balanced translocation. Male offspring that inherited the paternal copy of chromosome 7 missing the translocated region and both the normal chromosome 7 and the X chromosome with the chromosome translocation maternally were effectively maternally disomic for the PWS critical gene region. These mice exhibited suckling difficulties and postnatal lethality, resembling PWS infants.

A second PWS mouse model was created by targeted deletion of the PWS IC and exons 1-6 of *Snrpn* (Yang *et al.*, 1998). Examination of paternally expressed genes in the PWS region including *Mkrn3*, *Ndn*, and *Ipw* revealed an absence of expression confirming that the PWS-IC is required for normal expression of PWS candidate genes. These IC-deletion mice displayed reduced size, mild hypotonia, failure to thrive, and neonatal lethality between 5 and 8 days after birth. A subsequent study demonstrated that the neonatal lethality of the IC-

deletion mice to be dependent on strain background (Chamberlain *et al.*, 2004), and though no severe phenotypes were observed in surviving mice, they remain severely runted throughout life (Chamberlain *et al.*, 2004, Relkovic *et al.*, 2010). This model also demonstrates altered editing of the serotonin 2C receptor ($5HT_{2C}R$) pre-RNA, and alterations in behaviour associated with this receptor, including increased impulsivity and food seeking (Doe *et al.*, 2009). The authors attribute these findings to loss of the *Snord115* snoRNA cluster, because *SNORD115* has been associated with altered editing of $5HT_{2C}R$ in people with PWS (Kishore and Stamm, 2006), however, loss of the other PWS candidate genes in this model cannot be ruled out as contributing factors. Further behavioural changes have been described in this model, including reduced locomotion and impaired attention (Relkovic *et al.*, 2010), highlighting a role for the PWS region in behaviour.

Another model lacking expression of the full PWS critical region was created by insertion of a large transgene containing the Epstein-Barr virus Latent Membrane Protein 2A into the mouse chromosome 7 syntenic region, resulting in a deletion of the entire PWS/AS homologous region. This model produces models of both PWS and AS depending on the sex of the parent animal (Gabriel *et al.*, 1999). Mice that inherit the transgene paternally show reduced movement, growth, and respiratory problems, and typically die within one week of birth. Though these models are phenotypically similar with respect to failure to thrive phenotypes, the early lethality prevented in depth study into the PWS phenotype

in these model systems, necessitating the generation of single-gene targeted models.

Ndn-null mouse models

Four different mouse models missing expression of necdin have been generated. The first *Ndn*-null mouse model described replaced most of the *Ndn* sequence with a beta-galactosidase reporter gene (Tsai *et al.,* 1999). These mice are overtly normal, viable, fertile, and do not develop obesity. A second *Ndn*-null mouse model described was created by the insertion of a lacZ transgene into the necdin open reading frame (Gerard *et al.,* 1999). These *Ndn*-null mice display 100% neonatal lethality on a C57Bl/6J background, attributed to respiratory defects originating in the respiratory rhythm generator known as the pre-Bötzinger complex (Ren *et al.,* 2003, Pagliardini *et al.,* 2005). Reduced innervation of sympathetic nervous system targets, and impaired neuronal migration have also been observed in this model (Tennese *et al.,* 2008, Miller *et al.,* 2009). The third *Ndn*-null mouse model replaced the *Ndn* promoter region and most of the open reading frame with a neomycin cassette (Muscatelli *et al.,* 2000). These *Ndn*-null mice showed early postnatal lethality of partial penetrance, and survivors go on to display several phenotypes including increased skin picking behaviour, improved spatial learning and memory, and reduced gonadotropin releasing hormone (GnRH) and OT neuron numbers. Additionally, these mice show higher rates of apoptosis in developing neurons (Andrieu *et al.,* 2006). The fourth *Ndn*-null model was created by insertion of a Pgk1/Neomycin reporter cassette into the *Ndn* open reading frame. These mice display pain insensitivity,

increased apoptosis in dorsal root ganglia (Kuwako *et al.*, 2005), cerebellar granule neurons (Kurita *et al.*, 2006), and cortical neurons (Hasegawa and Yoshikawa, 2008), as well as defects in the differentiation and migration of GABAergic neurons in the brain (Kuwajima *et al.*, 2006, Kuwajima *et al.*, 2010). Taken together, the results from these *Ndn*-null models suggest an important role for necdin in the regulation of normal brain development, especially through the regulation of neuronal differentiation, apoptosis, and migration.

SnoRNA deletion models

A large, radiation-induced deletion that includes the *Snord115* locus did not result in any notable phenotype in mice (Ding *et al.*, 2005). Two different *Snord116*-null mice have been described. The first used a Cre-lox strategy to delete the chromosome 7 region including the *Snord116* gene cluster and several exons of the *Ipw* locus (Skryabin *et al.*, 2007). These mice display partial neonatal lethality, and postnatal growth retardation. The second *Snord116*-deletion mouse model was also created using a Cre-lox strategy. These mice also display postnatal growth retardation, and also demonstrate delayed sexual maturation, increased food intake, increased anxiety, and deficient motor learning (Ding *et al.*, 2008), supporting a role for *Snord116* in several major PWS phenotypes. Importantly, none of the mouse models described thus far recapitulate the predominant findings of hyperphagia and obesity seen in PWS, indicating a different PWS candidate gene or the interaction of multiple PWS genes is responsible for this phenotype.

Magel2-null mouse models

The first *Magel2*-null mouse model was created by inserting a lacZ transgene into the *Magel2* open reading frame (Kozlov *et al.*, 2007). These mice are the subject of the data chapters that follow in this thesis, and present numerous PWS-like phenotypes. Initially, these mice were demonstrated to have notable circadian rhythm defects, and dramatically reduced activity levels (Kozlov *et al.*, 2007). Reduced numbers of orexin (ORX) neurons in the lateral hypothalamus, and subsequent reductions in ORX levels were noted. Subsequent study demonstrated growth abnormalities reminiscent of PWS, with reduced weight gain in early life, followed by excessive weight gain and increased adiposity in adulthood (Bischof *et al.*, 2007). This *Magel2*-null mouse remains the only PWS mouse model to demonstrate an obese phenotype.

Chapters of this thesis will describe neuroanatomic and behavioural alterations ((Mercer *et al.*, 2009), Chapter 2), reproductive impairment ((Mercer and Wevrick, 2009), Chapter 3), and energy balance abnormalities (Chapter 4) in this *Magel2*-null mouse model, clearly demonstrating a role for *Magel2* in numerous PWS phenotypes. A second *Magel2*-null mouse has recently been described, using the Cre-lox strategy to delete the *Magel2* promoter and part of the open reading frame (Schaller *et al.*, 2010). These mice display a severe postnatal lethality due to feeding defects that is rescued with OT injection, suggesting a role for *Magel2* in feeding difficulties and failure to thrive in PWS. Like the original *Magel2*-null mouse strain, these mice have reductions in hypothalamic ORX levels, but in contrast, they also show reductions in

hypothalamic AVP and OT, though pituitary levels of these neuropeptides are not different between *Magel2*-null and control mice. The authors mention, but do not describe, circadian rhythm defects, infertility, and behavioural differences in their *Magel2*-null mice (Schaller *et al.*, 2010), suggesting significant overlap between the two *Magel2*-null mouse strains. Reported differences in neuropeptide levels and postnatal lethality in the *Magel2*-null mice could be a result of strain differences (C57Bl/6 vs 129Sv/Pas), or the *Magel2* targeting strategy, with the later model deleting the *Magel2* promoter, and the earlier model keeping the promoter intact.

1.2 The Hypothalamus

Numerous features of PWS have been related to hypothalamic defects including short stature/GH deficiency, hyperphagia/lack of satiety, hypogonadism, sleep disturbances, central adrenal insufficiency, and thermoregulation defects. Though it is poorly understood how individual PWS candidate genes contribute to hypothalamic development and function, the two PWS candidate genes with highest hypothalamic expression during development are *Necdin* and *Magel2* (Lee *et al.*, 2003). This thesis will highlight the roles of *Magel2* in neurochemical, reproductive, and energy balance regulation functions of the hypothalamus.

1.2.1 Development and Structure of the Hypothalamus

The hypothalamus develops from the ventral diencephalon and can be identified as a specific cell population as early as E9.5 in the mouse brain (Szabo *et al.*, 2009). Regulation of Sonic Hedgehog and Nodal signaling are critical for

early hypothalamic development and patterning (Szabo *et al.*, 2009, Mathieu *et al.*, 2002, Manning *et al.*, 2006), with Nodal signals required for proper development of the posterior-ventral region, and Hedgehog signaling favouring the anterior-dorsal hypothalamus (Mathieu *et al.*, 2002). Generation of the major nuclei of the hypothalamus takes place between E11 and E15 (Markakis., 2002), and various transcription factor pathways are critical for the development of these nuclei, including *Sim1, Otp, Nkx2.1, Sf1, Hmx2/Hmx3, Mash1,* and *Sox3* (Szarek *et al.*, 2010). Together these transcription factors provide a rudimentary hypothalamic transcription factor code allowing for the identification of hypothalamic cell types during development.

The vertebrate hypothalamus is a small region of the brain bordered anteriorly by the optic chiasm and posteriorly by the mammillary body, and is dorsal to the pituitary gland and ventral to the thalamus. The hypothalamus is the region of the brain most important for maintaining homeostasis, and is subdivided into 11 discrete nuclei which perform distinct functions that will be discussed in detail later. The hypothalamus can be divided into three longitudinally oriented zones (medial to lateral), the periventricular, medial, and lateral zones. The periventricular zone is located immediately adjacent to the third ventricle and is important for autonomic and neuroendocrine regulation. The medial zone is situated immediately adjacent to the periventricular zone and also plays important roles in autonomic and neuroendocrine regulation. Finally, the lateral zone, which is most clearly demarcated by the fornix, is also involved in autonomic regulation.

These zones can be further subdivided into 4 regions based on anterior-posterior location: preoptic, anterior, tuberal, and mammillary (Toni *et al.*, 2004).

Hypothalamic nuclei are bilaterally symmetrical, discrete structural groupings of neurons with specialized functions and can be grouped according to their locations in the various hypothalamic zones and regions (Figure 1.2). The arcuate nucleus (ARC) extends from the periventricular zone laterally into the medial zone, and is located on the floor of the tuberal region. The paraventricular nucleus (PVN) is located in the periventricular zone, and runs from the anterior posteriorly to the tuberal region. The supraoptic nucleus (SON) is found immediately above the optic chiasm in the medial to lateral hypothalamic zones, in the anterior region. The suprachiasmatic nucleus (SCN) is located on the floor of the anterior region of the medial zone, below the anterior hypothalamic area (AHA). The preoptic area (POA) is also located in the medial zone of the hypothalamus and extends into the lateral zone in the preoptic region. Nuclei found in the tuberal region of the medial zone include the ventromedial (VMN), and dorsomedial (DMN) nuclei. The posterior hypothalamic nucleus (PHN), and the mammillary nucleus (MN) are found in the mammillary region of the medial zone, and the lateral hypothalamus (LH), which is less clearly defined than other hypothalamic nuclei, is found in the lateral zone in the tuberal region extending into the mammillary region.

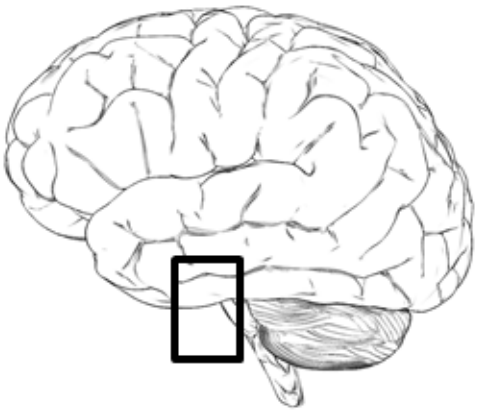
1.2.2 Functions of Hypothalamic Nuclei

As mentioned above, the overall function of the hypothalamus has long been associated with the maintenance of homeostasis (Bernard., 1974 [1878]).

This section will outline in greater detail specific functions of the individual hypothalamic nuclei, which are indicated in Table 1.4.

Preoptic Area (POA)

The POA is often subdivided into medial and lateral regions. The medial POA (mPOA) plays a very important role in the regulation of sexual behaviour. It is here where the majority of GnRH (also known as luteinizing hormone releasing hormone/LHRH) neurons reside (Naik., 1975, Ibata *et al.*, 1979, King *et al.*, 1985), releasing GnRH into the median eminence (ME), where it acts on the anterior pituitary gland to stimulate the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH). Furthermore, this region of the POA works with the lateral POA (lPOA) to play an important role in the generation of thirst (Cambiasso and Chiaraviglio, 1992, Osaka *et al.*, 1993) via noradrenaline secretion (Miyakubo *et al.*, 2003). The lPOA has also been shown to play an important role in sleep regulation, specifically the control of non-REM sleep (Kubota *et al.*, 2002), at least in part by communicating with other hypothalamic neurons involved in sleep and arousal (Suntsova *et al.*, 2007).



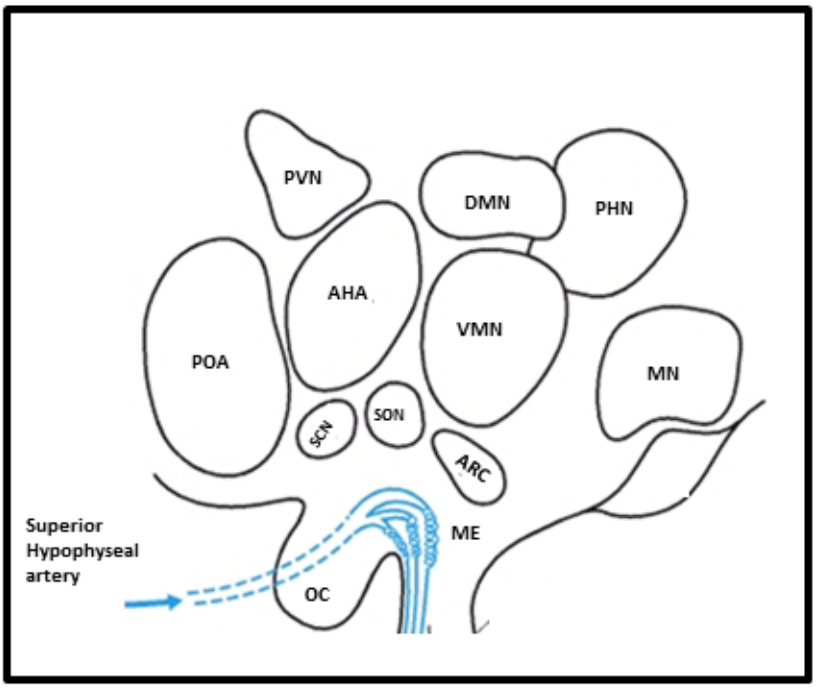

PVN
DMN
PHN
AHA
VMN
POA
MN
SCN
SON
ARC
Superior
Hypophyseal
artery
ME
OC

Figure 1.2 Organization of Hypothalamic Nuclei

Upper: Sagittal view of the brain outlining the location of the hypothalamus. Lower: Specific locations of hypothalamic nuclei. POA, preoptic area; SCN, suprachiasmatic nucleus; AHA, anterior hypothalamic area; SON, supraoptic nucleus; PVN paraventricular nucleus; ARC arcuate nucleus; VMN ventromedial nucleus; DMN, dorsomedial nucleus; PHN, posterior hypothalamic nucleus; MN, mammillary nucleus; OC, optic chiasm; ME, median eminence. *Adapted from (Nussey and Whitehead, 2001).*

Table 1.4 Functions of Hypothalamic Nuclei

Preoptic area	POA	Sexual behaviour, GnRH production, thirst, non-REM sleep onset,
Suprachiasmatic nucleus	SCN	Circadian rhythm
Anterior hypothalamic area	AHA	Thermoregulation, blood pressure
Supraoptic nucleus	SON	Fluid balance regulation - production of AVP, reproduction - production of OT
Paraventricular nucleus	PVN	Production of OT, production of AVP, production of CRH, production of TRH, regulation of appetite
Arcuate nucleus	ARC	Regulation of appetite and metabolism, reproduction, GHRH release
Ventromedial nucleus	VMN	Satiety, sexual behaviour, circadian rhythm modulation?
Dorsomedial nucleus	DMN	Body weight regulation, circadian rhythm, stress response
Posterior hypothalamic nucleus	PHN	Thermoregulation, wakefulness
Mammillary Nucleus	MN	Emotion, short-term memory
Lateral hypothalamus	LH	Food intake, reward and motivation, wakefulness

Suprachiasmatic Nucleus (SCN)

In the early 1970's two independent groups identified the SCN as a critical area involved in the regulation of circadian rhythms (Moore and Eichler, 1972, Stephan and Zucker, 1972). Since that time, a very large body of research has been compiled describing how this small grouping of approximately 20,000 neurons controls circadian rhythm in numerous species (for reviews see e.g. (Weaver., 1998, Kwon *et al.*, 2011)). Importantly, the SCN has also been shown to control the rhythms generated in peripheral tissues as well (Bernard *et al.*, 2007, Mohawk *et al.*, 2012), further underlining its role in body rhythm generation and control.

Anterior Hypothalamic Area (AHA)

The AHA is often grouped together with the POA in playing a key role in thermoregulation (Onoe *et al.*, 1992, Boulant., 2000). The AHA also plays a role in blood pressure regulation, with AHA lesions causing increases in arterial pressure (Wyss *et al.*, 1990) and increases in AHA neuronal activity being observed following changes in arterial pressure (Wyss *et al.*, 1999).

Supraoptic Nucleus (SON)

The SON is a small nucleus comprising about 300 magnocellular neurosecretory cells. The cell bodies of the SON produce either arginine vasopressin (AVP) or OT. Each SON neuron has a long axon that projects to the posterior pituitary gland, where AVP and OT are stored prior to their release into the blood stream. AVP plays an important role in fluid balance regulation, and its secretion is modulated through changes in osmosensitive currents in the SON

(Zhang *et al.*, 2009, Zhang *et al.*, 2007). OT plays an important role in reproduction, and is involved in mating, partuition, and milk let down (Bealer *et al.*, 2010, Borrow and Cameron, 2012). The SON receives considerable input from the SCN which is believed to regulate diurnal changes in AVP and/or OT secretion (Cui *et al.*, 1997).

Paraventricular Nucleus (PVN)

The PVN is an integrative hypothalamic nucleus, containing magnocellular neurosecretory cells and parvocellular neurosecretory cells that project to the posterior pituitary and median eminence respectively, and a number of other neurons expressing various peptides that project throughout the brain. Like those of the SON, the PVN magnocellular neurons release both OT and AVP, playing a role in reproductive and osmoregulatory control. PVN parvocellular neurons project to the ME, where they release corticotropin releasing factor (CRF) and thyrotropin releasing hormone (TRH), which control the pituitary secretion of adrenocorticotropic hormone (ACTH), and thyroid stimulating hormone (TSH) respectively. Centrally-projecting neurons of the PVN include parvocellular OT cells which project to the brain stem and spinal cord playing roles in gastric reflexes (Calatayud *et al.*, 1999) and penile erection (Melis and Argiolas, 2011), parvocellular AVP cells that project throughout the hypothalamus and also to the brain stem playing roles in thermoregulation (Madden and Morrison, 2009) and blood pressure regulation (Kc *et al.*, 2010), and parvocellular CRF cells which likely play a role in stress responses (Busnardo *et al.*, 2010, Greetfeld *et al.*, 2009). The PVN also plays a key role in energy balance

regulation, receiving direct projections from neurons in the ARC that respond to circulating signals of energy balance. Stimulation of the PVN causes a reduction in food intake, and PVN lesions cause obesity. PVN actions on energy balance have at least partially been shown to be mediated by CRH (Glowa *et al.*, 1992) and TRH and its metabolites (Kow and Pfaff, 1991).

Arcuate Nucleus (ARC)

The ARC contains both neuroendocrine neurons and centrally projecting neurons. Neuroendocrine neurons of the ARC produce either dopamine or GHRH, and release them into the ME where they act on the anterior pituitary. There, dopamine inhibits prolactin secretion suppressing milk production (Freeman *et al.*, 2000), and GHRH stimulates the release of GH, stimulating anabolism (Bluet-Pajot *et al.*, 1998). Centrally projecting ARC neurons include first order neurons responsible for energy balance regulation, namely those that produce the orexigenic neuropeptides NPY and AgRP, and those that produce the anorexigenic neuropeptides pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART). Both NPY/AgRP and POMC/CART neurons respond directly to circulating signals of energy balance including leptin and insulin (Leibowitz and Wortley, 2004). These neurons send dense projections to the PVN to ultimately regulate food intake and metabolic expenditure (Morton *et al.*, 2006). Other centrally-projecting neurons in the ARC express kisspeptin, which acts directly on GnRH neurons to stimulate GnRH secretion (Messager *et al.*, 2005, d'Anglemont de Tassigny *et al.*, 2008). The kisspeptin neurons also inhibit ARC dopamine neurons to regulate prolactin release (Szawka *et al.*, 2010).

Ventromedial Nucleus (VMN)

The VMN plays a key role in satiety, with VMN disruption leading to hyperphagia from both increased meal size and number (King., 2006). The VMN has also been implicated in the control of sexual behaviour, inhibiting lordosis behaviour in female rats (Matsumoto and Yamanouchi, 2000), and inducing motivational components of male sexual behaviour through androgen activation (Harding and McGinnis, 2003, Harding and McGinnis, 2005). The VMN also appears to play a role in the phase regulation of circadian corticosterone rhythm (Egawa *et al.*, 1991), and temperature and activity rhythms related to food seeking (Challet *et al.*, 1997).

Dorsomedial Nucleus (DMN)

The DMN is involved in the regulation of food intake, with DMN lesions causing hypophagia. The DMN is a site where opioids and glucose act to stimulate food intake, and is also the area responsible for the feeding response to an imbalanced diet (Bellinger and Bernardis, 2002). The DMN plays a role in circadian rhythm and receives both direct and indirect inputs from the SCN (Chou *et al.*, 2003). The DMN plays an especially important role in food entrainable circadian rhythms, sending direct projections to orexin neurons in the lateral hypothalamus which are known to be required in the expression of food entrained behavioural rhythms (Gooley *et al.*, 2006, Mieda *et al.*, 2006). Recently, the DMN has been shown to play a primary role in the synaptic integration underlying the cardiovascular response to emotional stress including increases in heart rate and mean arterial pressure (Fontes *et al.*, 2011).

Posterior Hypothalamic Nucleus (PHN)

The PHN plays a role in thermoregulation by evoking shivering thermogenesis (Thornhill and Halvorson, 1994). The PHN is also an important hypothalamic area in wakefulness, and restoration of orexin 2 receptors (OX2R) in the PHN and adjacent mammillary bodies rescues the sleepiness seen in OX2R null narcoleptic mice (Mochizuki *et al.*, 2011).

Mammillary Nucleus (MN)

The MN region of the hypothalamus plays a role in spatial memory, specifically short-term memory, through direct and indirect connections to the temporal lobe (Vann and Aggleton, 2004). The MN has been implicated in fear conditioning (Conejo *et al.*, 2007) and may also play a role in wakefulness via OX2R expression (Mochizuki *et al.*, 2011).

Lateral Hypothalamus (LH)

The LH has long since been thought of as a hunger centre, with lesions of the LH leading to starvation (Anand and Brobeck, 1951). Cell bodies of ORX producing neurons are found in the LH, ORX having known roles in stimulation of food intake and wakefulness. Another important function of the LH is reward and motivation which is also mediated through orexin neurons (Harris *et al.*, 2005, Aston-Jones *et al.*, 2010).

1.2.3 Expression of Magel2 in the Hypothalamus

Magel2 expression is localized to the brain in both embryos and adults (Lee *et al.*, 2000, Boccaccio *et al.*, 1999), with highest levels of expression in the developing mouse brain seen at E15-E17. Expression during brain development is

highest in hypothalamic regions, with highest levels noted in the SCN, SON, and POA. High levels are also seen in the ARC and PVN (Lee *et al.*, 2003). A very similar expression pattern is seen in the adult hypothalamus (Figure 1.3). Highest levels of *Magel2* are seen in the SCN and PVN, with very high expression also seen in the SON, ARC, VMN, and DMN. Notable levels of *Magel2* expression are also visible throughout the POA (Lein *et al.*, 2007). Based on the expression pattern of *Magel2*, it seems likely that this gene could very well play a role in PWS phenotypes such as those related to sleep disturbances (SCN), reproductive function (ARC, POA), and energy homeostasis (ARC, PVN, VMN, DMN). As previously mentioned, *Magel2*-null mice do indeed show circadian rhythm disturbances (Kozlov *et al.*, 2007), and alterations in body weight gain (Bischof *et al.*, 2007). This thesis will describe alterations in behaviour, fertility, and energy balance regulation in the *Magel2*-null mouse, confirming its likely contribution to these PWS phenotypes.

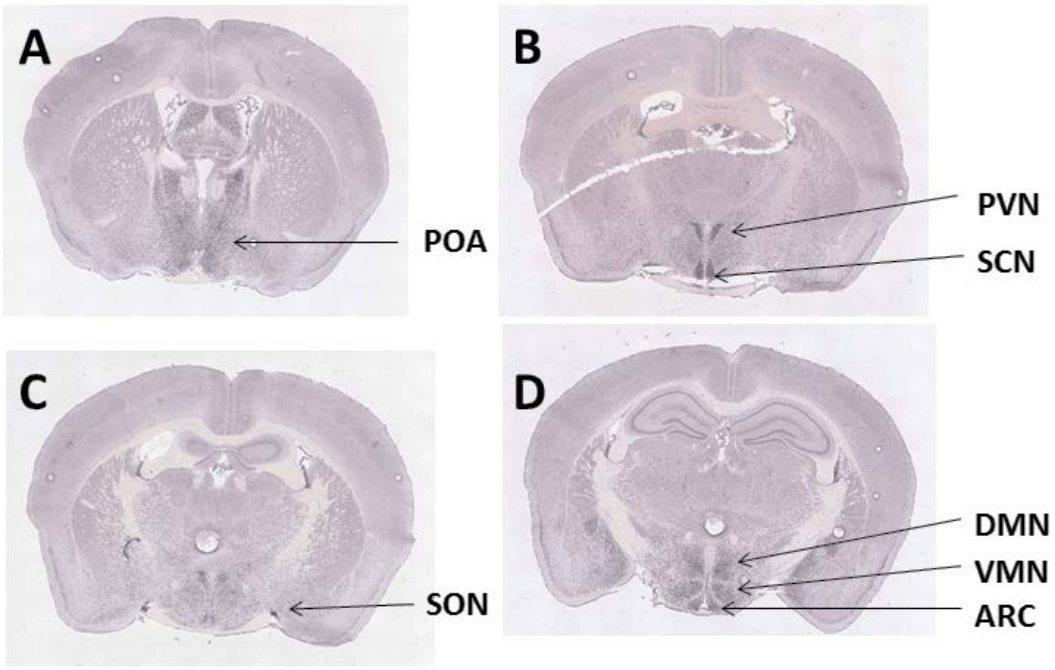


Figure 1.3 *Magel2* Expression in the Adult Mouse Hypothalamus

In situ hybridization of *Magel2* expression in coronal sections of the mouse brain. A-D rostral-caudal. POA, preoptic area; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; DMN, dorsomedial nucleus; VMN, ventromedial nucleus; ARC, arcuate nucleus. *Images downloaded from Allen Mouse Brain Atlas [Internet]. Seattle (WA): Allen Institute for Brain Science. ©2009. Available from: http://mouse.brain-map.org.*

1.3 Regulation of Fertility by the Hypothalamus

1.3.1 The HPG Axis

Normal sexual development and maintenance of fertility requires proper development and function of the hypothalamic-pituitary-gonadal (HPG) axis (Figure 1.4). Briefly, GnRH (also known as LHRH) is produced in the hypothalamus, where it is released into the hypophyseal portal blood stream via the ME (see Figure 1.2) and is carried to the anterior pituitary gland where it acts on gonadotroph cells that express the specific GnRH-receptor (Millar., 2005). GnRH stimulates the gonadotrophs to produce and release FSH and LH, which act on the gonads to stimulate the production of androgens and estrogens, which feedback both at the levels of the hypothalamus and the pituitary to inhibit the production of GnRH and LH/FSH respectively (Vadakkadath, Meethal, and Atwood, 2005). The HPG axis is critical in controlling puberty, fertility, and reproductive senescence, and impairments at any level (brain, pituitary, gonad) can impair these functions.

The initiation of puberty is a result of heightened amplitudes of GnRH pulsation, which activates the HPG axis, stimulating production of estrogen from the ovaries and testosterone from the testes (Mauras *et al.*, 1996). The cause of increased GnRH pulses has been linked to increases in circulating signals, with leptin thought to play a key role (Mauras *et al.*, 1996, Suter *et al.*, 2000, Elias., 2012). The conclusion of puberty is generally defined as the attainment of the ability to reproduce, with the presence of adult levels of sex hormones. The regulation of fertility through GnRH secretion differs in males and females, with

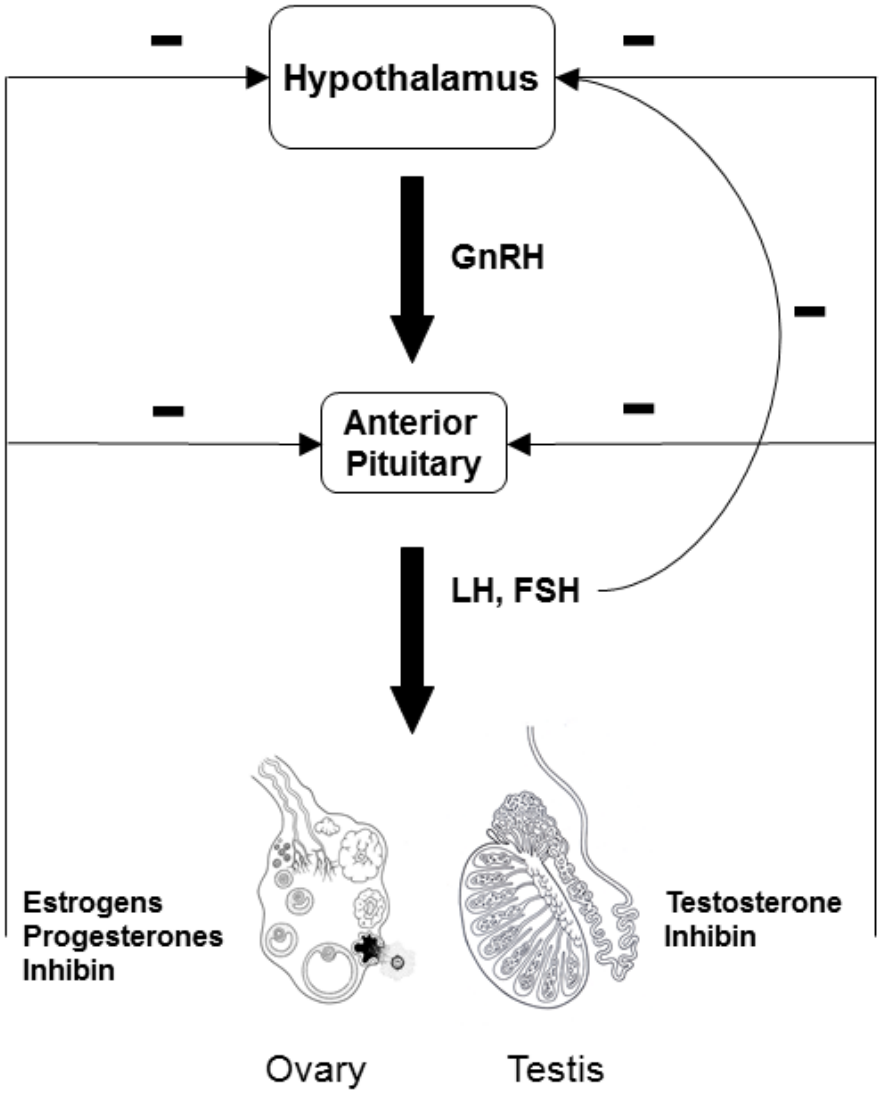


Figure 1.4 The Hypothalamic-Pituitary-Gonadal axis.

GnRH is produced by the hypothalamus and released into the hypophyseal portal blood stream, where it stimulates the pituitary to produce LH and FSH. LH and FSH act on the gonads to produce sex steroids, which form a negative feedback loop at both the level of the hypothalamus and the level of the pituitary gland to inhibit further LH/FSH production.

constant pulse frequency continuing through life in males, and varied pulse frequency in females controlling the estrous (in humans, menstrual) cycle, with large GnRH surges triggering ovulation. In males, LH stimulates the production of testosterone (Veldhuis *et al.*, 2009) and FSH plays an important role in spermatogenesis (Sofikitis *et al.*, 2008). In females, FSH controls follicular growth, with LH supporting theca cells, which produce hormonal precursors for estradiol production. An LH surge triggers ovulation of mature follicles, and LH is necessary to maintain function of the resulting corpus lutea (Palermo., 2007).

The activity of the HPG axis decreases with age in both males and females. In females, the availability of follicles is decreased and the ability of estrogen to act on GnRH neurons to stimulate the LH surge necessary for ovulation is diminished, leading to irregular cycles and the termination of reproductive function (Downs and Wise, 2009). Though males retain the potential for fertility, reduced GnRH pulsatility and reduced ability of LH to stimulate testosterone production lead to loss of libido and abnormal sperm production (Keenan and Veldhuis, 2009).

1.3.2 Origin and Regulation of GnRH Neurons

Proper migration and function of GnRH neurons is critical for the development and maintenance of fertility in mammals. Originating in the olfactory placode in mid-development, GnRH neurons undertake a lengthy migration through the brain to their final location in the basal hypothalamus. In rodent species, GnRH neurons are found distributed within the medial septum, POA, and AHA, with the majority of cells seen in the mPOA. GnRH neurons are

also found as caudal as the ARC in sheep and primates (Jasoni *et al.*, 2009). GnRH neurons in the POA are the most crucial for maintaining reproductive functions, including regulating puberty (Clarkson and Herbison, 2006), and generating the preovulatory LH surge (Wintermantel *et al.*, 2006).

In mice, GnRH neurons are generated in the olfactory placode between E9.5 and E12.5 (Wray *et al.*, 1989, Schwanzel-Fukuda and Pfaff, 1989). GnRH gene expression is initiated at E11.5 (Schwanzel-Fukuda and Pfaff, 1989), and has been used to follow the subsequent migration of GnRH neurons through the brain. The GnRH neurons first migrate through the nasal compartment, in association with olfactory/vomeronasal axons in association with numerous guidance cues (reviewed in (Wray., 2001)). They then cross the cribriform plate and penetrate the brain near the olfactory bulbs, and follow a caudally turning pathway towards the developing hypothalamus (Wray *et al.*, 1994). Finally, the GnRH neurons continue to migrate with a transient projection of the vomeronasal nerve in a caudal and ventral direction towards the basal forebrain (Yoshida *et al.*, 1995). The GnRH neurons dissociate from the guiding axons of the vomeronasal nerve and take up their final positions in the hypothalamus, where they form projections into the ME (Cariboni *et al.*, 2007).

Factors that impair the birth, migration, and projection of GnRH neurons lead to infertility and/or hypogonadism in humans. For example, anosmin, the product of the KAL-1 gene which causes Kallman syndrome in people when disrupted, is critical for olfactory and GnRH neuron migration (Ballabio and Camerino, 1992) and appears to halt GnRH neuron migration at the cribriform

plate (Hu and Bouloux, 2011). Interestingly, the PWS candidate gene Necdin has been shown important in the birth and final projection of GnRH neurons towards the ME, thus likely playing a role in the hypogonadism phenotype in PWS individuals (Miller *et al.*, 2009).

It has previously been mentioned that GnRH neurons are negatively regulated by the sex steroids (estrogen, testosterone, and inhibin) produced under control of the HPG axis. Many other factors, both physiological and environmental regulate these neurons to modulate fertility as well. For example, nutritional status has a profound role in maintenance of fertility, with both excess- and under-nutrition causing negative regulation of the HPG axis (Hill *et al.*, 2008, Schneider., 2004). The effects of nutrition on fertility are indirect on GnRH neurons. As an example, the adipose derived hormone leptin acts on kisspeptin neurons on the ARC, which then project directly onto GnRH neurons (Castellano *et al.*, 2010). The leptin-kisspeptin-GnRH connection is especially important in the progression of puberty (Roa *et al.*, 2009), and the inhibition on GnRH neurons during pregnancy and lactation (Smith *et al.*, 2010). In seasonally breeding animals, photoperiod plays a key role in the stimulation and inhibition of GnRH neurons, and this regulation has also been related to ARC kisspeptin neurons (Smith., 2011, Ansel *et al.*, 2011). Dysregulation of circadian rhythm has notable impact on reproductive function, and is discussed in Chapter 3 of this thesis.

1.4 Hypothalamic Regulation of Energy Balance

Energy balance refers to the process whereby energy reserves are maintained over a long period of time. Energy input, in the form of nutrient intake

is balanced by energy expenditure from basal metabolism, thermogenesis, and physical activity. The hypothalamus has emerged as a major centre for the homeostatic regulation of energy balance, though numerous other brain areas play a role in motivation and reward, as well as social influences and their impact on food intake (Berthoud and Morrison, 2008). Numerous hypothalamic peptides play key roles in energy balance regulation, and the expression of these peptides is controlled by peripheral energy balance signals. Hypothalamic peptides and peripheral signals involved in energy balance regulation are briefly discussed below.

1.4.1 Hypothalamic Peptides Involved in Energy Balance Regulation

Key regions of the hypothalamus involved in energy balance regulation include the ARC, PVN, and LH. In these regions neurons express numerous orexigenic and anorexigenic peptides which act on other brain regions to control food intake and energy expenditure (Table 1.5).

ARC Peptides

The ARC is the region of the hypothalamus that directly senses circulating signals of energy balance. Because they directly respond to circulating signals such as insulin, leptin, and ghrelin (Figure 1.5), neurons of the ARC are often referred to as first-order neurons in the control of energy balance. The ARC receives both leptin and insulin through specialized transporters (Bouret., 2008, Banks., 2004), whereas ghrelin and other signals of energy balance likely act on the ARC *via* neuronal projections from circumventricular organs of the brain which are areas with fenestrated blood-brain barriers (Fry and Ferguson, 2007, Fry and Ferguson, 2010). The ARC contains two major populations of neurons

related to energy homeostasis: those that co-express the orexigenic peptides NPY and AgRP (Hahn et al., 1998), and those that co-express the anorexigenic peptides POMC, which is processed in these cells to produce α-melanocyte-stimulating hormone (α-MSH), and CART (Hahn *et al.*, 1998, Elias *et al.*, 2001). Both the NPY/AgRP and POMC/CART neurons send projections within the hypothalamus to the PVN (Baker and Herkenham, 1995) and LH (Elias *et al.*, 1998), areas housing second-order energy balance neurons. The ARC has also been shown to have a population of neurons that express ghrelin (Kageyama *et al.*, 2008, Cowley *et al.*, 2003), an orexigenic peptide mainly produced in the stomach that activates NPY/AgRP neurons (Cowley *et al.*, 2003, Andrews *et al.*, 2008). Also, the anorexigenic galanin-like peptide (GALP) is synthesized in the ARC (Shioda *et al.*, 2011).

PVN Peptides

PVN peptides involved in the regulation of energy balance include CRF and TRH. CRF expression increases in the presence of α-MSH (Fekete *et al.*, 2000b). CRF mediates its anorexigenic effects by reducing food intake, stimulating brown fat thermogenesis, and increasing locomotor activity (Uehara *et al.*, 1998, LeFeuvre *et al.*, 1987, Buwalda *et al.*, 1998).

Table 1.5 Hypothalamic Peptides Involved in the Control of Energy Balance

Peptide	Location of Synthesis	Reference
Orexigenic		
Neuropeptide Y (NPY)	ARC	A
Agouti-related peptide (AgRP)	ARC	B
Melanin concentrating hormone (MCH)	LH	C
Galanin	PVN	D
Orexin	LH	E
Ghrelin*	ARC (*stomach)	F
Anorexigenic		
Pro-opiomelanocortin (POMC)	ARC	G
Cocaine and amphetamine regulated transcript (CART)	ARC	H
Galanin like peptide (GALP)	ARC	I
Thyrotropin releasing hormone (TRH)	PVN	J
Corticotropin releasing factor (CRF)	PVN	K

Reference Review Articles: A (Mercer et al., 2011), B (Ilnytska and Argyropoulos, 2008), C (Griffond and Risold, 2009), D (Fang et al., 2011), E (Tsujino and Sakurai, 2009), F (Kageyama et al., 2010), G (Xu et al., 2011),H (Rogge et al., 2008), I (Lawrence and Fraley, 2011), J (Lechan and Fekete, 2006), K (Zorrilla et al., 2003).

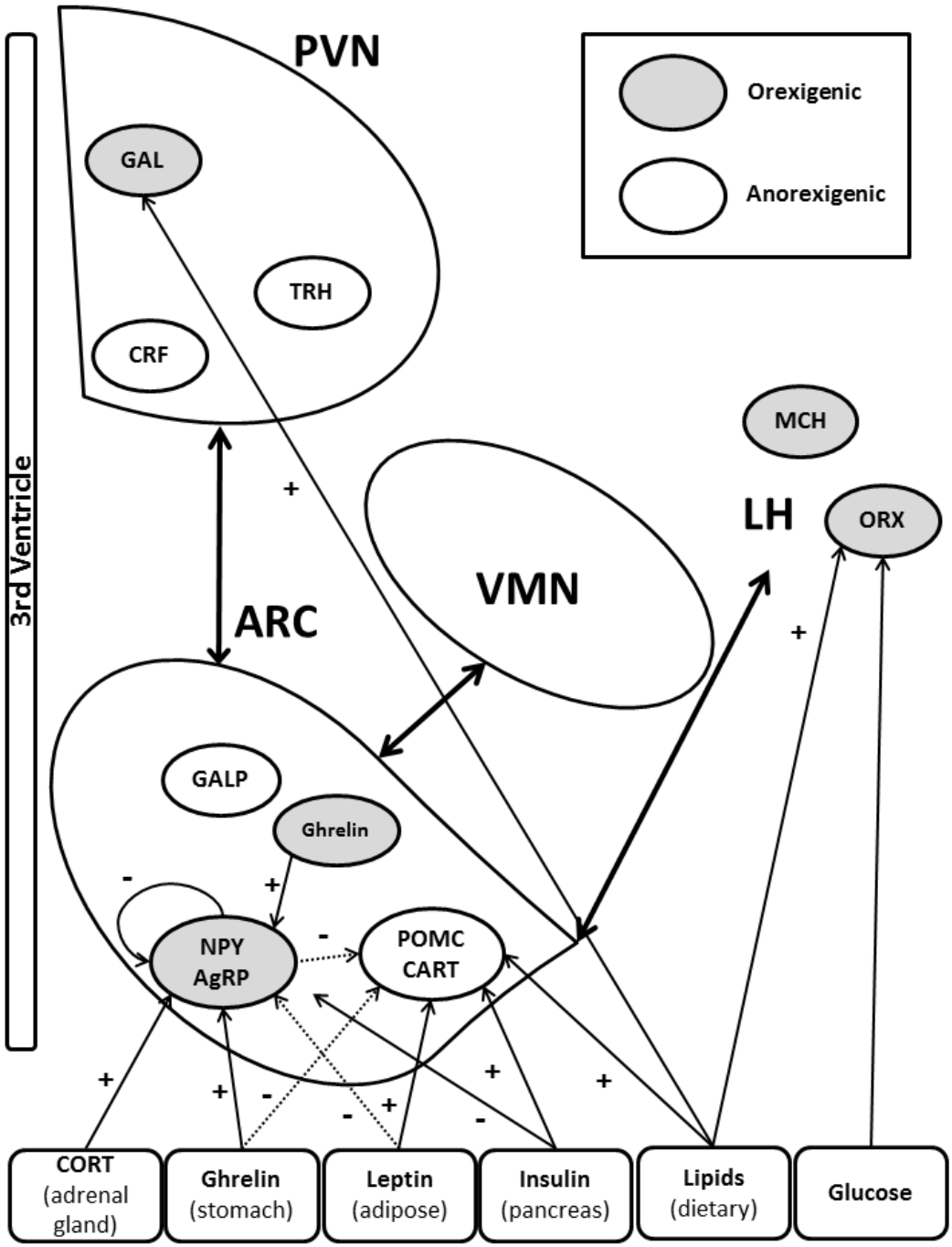

PVN
Orexigenic
Anorexigenic
GAL
TRH
CRF
3rd Ventricle
MCH
LH
ORX
VMN
ARC
GALP
Ghrelin
NPY
AgRP
POMC
CART
CORT
(adrenal
gland)
Ghrelin
(stomach)
Leptin
(adipose)
Insulin
(pancreas)
Lipids
(dietary)
Glucose

Figure 1.5 Model of Hypothalamic Peptide Systems Involved in Energy Balance Regulation

Peripheral signals are shown at the bottom and control the expression of hypothalamic peptides, indicated by arrows as either stimulatory (+) or inhibitory (-). Dashed arrows indicate effects that may be indirect. Orexigenic peptides are represented by shaded ovals and include NPY, neuropeptide Y; AgRP, agouti-related peptide; ghrelin; GAL, galanin; MCH, melanin-concentrating hormone; and ORX, orexin. Anorexigenic peptides include POMC, pro-opiomelanocortin; CART, cocaine and amphetamine regulated transcript; GALP, galanin-like peptide; CRF, corticotropin releasing factor; and TRH, thyrotropin releasing hormone. Hypothalamic areas represented are the ARC, arcuate nucleus; PVN, paraventricular nucleus; LH, lateral hypothalamus; and VMH, ventromedial hypothalamus. Reciprocal connections between hypothalamic areas are indicated by double headed arrows.

TRH expression and release are stimulated by both α-MSH (Fekete *et al.*, 2000a) and CART (Fekete *et al.*, 2000c), and inhibited by both NPY (Fekete *et al.*, 2001) and AgRP (Fekete *et al.*, 2002), indicating that the TRH neurons are key second order neurons in the regulation of energy balance. TRH mediates its anorexigenic effects by stimulating the thyroid gland, which leads to reduced food intake and increased thermogenesis (Choi *et al.*, 2002).

LH Peptides

The LH has two distinct populations of neurons that produce orexigenic peptides: those that produce the orexins (ORX) (also known as hypocretins), ORX-A and ORX-B, and those that produce melanin-concentrating hormone (MCH). ORX stimulates food intake and wakefulness, and is thought to coordinate food seeking behaviour with wakefulness (Willie *et al.*, 2001, Sakurai., 2005). Though its role in food intake is unclear, mice missing MCH (Segal-Lieberman *et al.*, 2003) or its receptor (Marsh *et al.*, 2002) become hyperactive, suggesting that MCH suppresses energy expenditure.

1.4.2 Peripheral Energy Signals Targeting the Hypothalamus

Numerous peripheral signals circulate in the blood stream and act on the hypothalamus, to stimulate or inhibit the production of the previously discussed peptides that are involved in energy balance regulation (Figure 1.5). Leptin, a hormone secreted by adipose tissue, appears to be the most major peripheral hormone involved in regulating energy balance. In *in vitro* studies, leptin inhibits NPY/AgRP neurons, and activates POMC neurons, leading to reduced food intake and increased energy expenditure (Gautron and Elmquist, 2011, Benoit *et al.*,

2004). Another major peripheral signal of energy balance is insulin, produced by the pancreas. Like leptin, insulin acts on the ARC to inhibit food intake and increase energy expenditure (Benoit *et al.*, 2004). The only peripheral orexigenic hormone is ghrelin, a peptide hormone secreted by the stomach before meals to stimulate food intake (Cummings., 2006). Ghrelin is also synthesized in a discrete population of ARC neurons, and both central and peripheral ghrelin act largely by stimulating ARC NPY/AgRP neurons (Kageyama *et al.*, 2010). A number of other gut hormones are involved in meal initiation and termination, and have been shown to signal to the brain to reduce food intake. They include cholecystokinin, glucagon-like peptide 1, oxyntomodulin, pancreatic polypeptide, and peptide YY (Murphy and Bloom, 2006).

1.5 Hypothesis and Summary of Studies

When I first joined the Wevrick laboratory in 2006, I was asked to help create weight gain curves of *Magel2*-null mice both before and after weaning. This data collection contributed to the manuscript by Bischof *et al.* (2007), showing that the *Magel2*-null mice were underweight prior to weaning, and overweight as adults. While completing these measurements, I anecdotally noticed that the generation of pups from *Magel2*-null fathers seemed to take longer than the generation of pups from carrier males who had inherited their mutant *Magel2* allele from their mothers. I also found that I could often identify the *Magel2*-null animals by observation, as they appeared less reactive to examination and handling. During this time, a paper was published identifying a circadian rhythm defect and reduced activity levels in *Magel2*-null mice (Kozlov

et al., 2007), suggesting that *Magel2* likely plays a role in various PWS phenotypes. Based on these early findings, and on the expression patterns of *Magel2* within the hypothalamus (Figure 1.4), I hypothesized that *Magel2* would also be involved in behavioural and reproductive function, and likely played a key role in both short- and long-term energy balance regulation.

Along with another graduate student in the Wevrick laboratory, Erin Kwolek, I initiated a behavioural study of the *Magel2*-null mice to see if some of the abnormal behaviours I was seeing in these mice were quantifiable and associated with an anxious phenotype. Indeed, we found that the behaviour of *Magel2*-null mice is abnormal, and is accompanied by certain neurochemical differences and reductions in volume in several discrete brain regions (Mercer and Wevrick, 2009). These findings are described in Chapter 2.

I began an independent study of reproductive function in *Magel2*-null mice in parallel with the behavioural experiments, examining the initiation and progression of puberty, and adult reproductive function in these mice. I found that *Magel2*-null mice have a delay in both the entrance and progression of puberty, and impaired reproductive function leading to early infertility (Mercer and Wevrick, 2009). Because the gonads in both male and female *Magel2*-null mice were largely normal, it appears these reproductive defects are central in origin, and likely originating in the hypothalamus. These findings are described in Chapter 3.

Finally, I returned to studies relating to energy balance, with the knowledge that the hormone leptin plays important roles in the regulation of

behaviour (Asakawa *et al.*, 2003, Harvey., 2007) and fertility (Pralong *et al.*, 2002, Israel and Chua, 2010). I hypothesized that the weight gain and reproductive phenotypes observed in *Magel2*-null mice may be a result of disrupted leptin signaling in the hypothalamus. In experiments aimed at examining leptin function in *Magel2*-null mice, I have shown that the mutant animals are leptin resistant, and this leptin resistance precedes their development of obesity. Further, I have shown that impaired leptin function in the ARC of *Magel2*-null mice is limited to cells which are normally activated by leptin, and confirmed this impairment in POMC neurons of the ARC. These experiments are described in Chapter 4.

The results of previously published studies, and the information contained in this thesis clearly indicate a role for *Magel2* in numerous physiological functions, and suggest a role for *MAGEL2* in the pathogenesis of PWS. Further study to determine a direct cellular role for Magel2 is needed to more completely understand the contribution of this gene to the various phenotypes discovered in *Magel2*-null mice.

1.6 Bibliography

Aebischer,J., Sturny,R., Andrieu,D., *et al.* (2011) "Necdin protects embryonic motoneurons from programmed cell death." PLoS One **6**(9):e23764.

Akefeldt,A., Tornhage,C. J. and Gillberg,C. (1999) "'A woman with Prader-Willi syndrome gives birth to a healthy baby girl'." Dev.Med.Child Neurol. **41**(11):789-790.

Anand,B. K. and Brobeck,J. R. (1951) "Localization of a "feeding center" in the hypothalamus of the rat." Proc.Soc.Exp.Biol.Med. **77**(2):323-324.

Andrews,Z. B., Liu,Z. W., Walllingford,N., *et al.* (2008) "UCP2 mediates ghrelin's action on NPY/AgRP neurons by lowering free radicals." Nature **454**(7206):846-851.

Andrieu,D., Meziane,H., Marly,F., Angelats,C., Fernandez,P. A. and Muscatelli,F. (2006) "Sensory defects in Necdin deficient mice result from a loss of sensory neurons correlated within an increase of developmental programmed cell death." BMC Dev.Biol. **6**:56.

Ansel,L., Bentsen,A. H., Ancel,C., *et al.* (2011) "Peripheral kisspeptin reverses short photoperiod-induced gonadal regression in Syrian hamsters by promoting GNRH release." Reproduction **142**(3):417-425.

Asakawa,A., Inui,A., Inui,T., Katsuura,G., Fujino,M. A. and Kasuga,M. (2003) "Leptin treatment ameliorates anxiety in ob/ob obese mice." J.Diabetes Complications. **17**(2):105-107.

Aston-Jones,G., Smith,R. J., Sartor,G. C., *et al.* (2010) "Lateral hypothalamic orexin/hypocretin neurons: A role in reward-seeking and addiction." Brain Res. **1314**:74-90.

Baker,R. A. and Herkenham,M. (1995) "Arcuate nucleus neurons that project to the hypothalamic paraventricular nucleus: neuropeptidergic identity and consequences of adrenalectomy on mRNA levels in the rat." J.Comp.Neurol. **358**(4):518-530.

Ballabio,A. and Camerino,G. (1992) "The gene for X-linked Kallmann syndrome: a human neuronal migration defect." Curr.Opin.Genet.Dev. **2**(3):417-421.

Banks,W. A. (2004) "The source of cerebral insulin." Eur.J.Pharmacol. **490**(1-3):5-12.

Barker,P. A. and Salehi,A. (2002) "The MAGE proteins: emerging roles in cell cycle progression, apoptosis, and neurogenetic disease." J.Neurosci.Res. **67**(6):705-712.

Bealer,S. L., Armstrong,W. E. and Crowley,W. R. (2010) "Oxytocin release in magnocellular nuclei: neurochemical mediators and functional significance during gestation." Am.J.Physiol.Regul.Integr.Comp.Physiol. **299**(2):R452-8.

Bellinger,L. L. and Bernardis,L. L. (2002) "The dorsomedial hypothalamic nucleus and its role in ingestive behavior and body weight regulation: lessons learned from lesioning studies." Physiol.Behav. **76**(3):431-442.

Benoit,S. C., Clegg,D. J., Seeley,R. J. and Woods,S. C. (2004) "Insulin and leptin as adiposity signals." Recent Prog.Horm.Res. **59**:267-285.

Bernard,C. (1974 [1878])"Lectures on the phenomena common to animals and plants"Charles C. ThomasSpringfield, IL.

Bernard,S., Gonze,D., Cajavec,B., Herzel,H. and Kramer,A. (2007) "Synchronization-induced rhythmicity of circadian oscillators in the suprachiasmatic nucleus." PLoS Comput.Biol. **3**(4):e68.

Berthoud,H. R. and Morrison,C. (2008) "The brain, appetite, and obesity." Annu.Rev.Psychol. **59**:55-92.

Bischof,J. M., Stewart,C. L. and Wevrick,R. (2007) "Inactivation of the mouse Magel2 gene results in growth abnormalities similar to Prader-Willi syndrome." Hum.Mol.Genet. **16**(22):2713-2719.

Bluet-Pajot,M. T., Epelbaum,J., Gourdji,D., Hammond,C. and Kordon,C. (1998) "Hypothalamic and hypophyseal regulation of growth hormone secretion." Cell.Mol.Neurobiol. **18**(1):101-123.

Boccaccio,I., Glatt-Deeley,H., Watrin,F., Roeckel,N., Lalande,M. and Muscatelli,F. (1999) "The human MAGEL2 gene and its mouse homologue are paternally expressed and mapped to the Prader-Willi region." Hum.Mol.Genet. **8**(13):2497-2505.

Borrow,A. P. and Cameron,N. M. (2012) "The role of oxytocin in mating and pregnancy." Horm.Behav. **61**(3):266-276.

Boulant,J. A. (2000) "Role of the preoptic-anterior hypothalamus in thermoregulation and fever." Clin.Infect.Dis. **31 Suppl 5**:S157-61.

Bouret,S. G. (2008) "Crossing the border: developmental regulation of leptin transport to the brain." Endocrinology **149**(3):875-876.

Brunelli,S., Tagliafico,E., De Angelis,F. G., *et al.* (2004) "Msx2 and necdin combined activities are required for smooth muscle differentiation in mesoangioblast stem cells." Circ.Res. **94**(12):1571-1578.

Buiting,K., Saitoh,S., Gross,S., *et al.* (1995) "Inherited microdeletions in the Angelman and Prader-Willi syndromes define an imprinting centre on human chromosome 15." Nat.Genet. **9**(4):395-400.

Bush,J. R. and Wevrick,R. (2012) "Loss of the Prader-Willi obesity syndrome protein necdin promotes adipogenesis." Gene **497**(1):45-51.

Bush,J. R. and Wevrick,R. (2010) "Loss of Necdin impairs myosin activation and delays cell polarization." Genesis **48**(9):540-553.

Bush,J. R. and Wevrick,R. (2008) "The Prader-Willi syndrome protein necdin interacts with the E1A-like inhibitor of differentiation EID-1 and promotes myoblast differentiation." Differentiation **76**(9):994-1005.

Busnardo,C., Tavares,R. F., Resstel,L. B., Elias,L. L. and Correa,F. M. (2010) "Paraventricular nucleus modulates autonomic and neuroendocrine responses to acute restraint stress in rats." Auton.Neurosci. **158**(1-2):51-57.

Butler,M. G., Theodoro,M. F., Bittel,D. C. and Donnelly,J. E. (2007) "Energy expenditure and physical activity in Prader-Willi syndrome: comparison with obese subjects." Am.J.Med.Genet.A. **143**(5):449-459.

Butler,M. G. and Palmer,C. G. (1983) "Parental origin of chromosome 15 deletion in Prader-Willi syndrome." Lancet **1**(8336):1285-1286.

Buwalda,B., Van Kalkeren,A. A., de Boer,S. F. and Koolhaas,J. M. (1998) "Behavioral and physiological consequences of repeated daily intracerebroventricular injection of corticotropin-releasing factor in the rat." Psychoneuroendocrinology **23**(3):205-218.

Calatayud,S., Quintana,E., Esplugues,J. and Barrachina,M. D. (1999) "Role of central oxytocin in the inhibition by endotoxin of distension-stimulated gastric acid secretion." Naunyn Schmiedebergs Arch.Pharmacol. **360**(6):676-682.

Cambiasso,M. J. and Chiaraviglio,E. (1992) "The involvement of the hypothalamic preoptic area on the regulation of thirst in the rat." Behav.Neural Biol. **58**(3):190-195.

Cariboni,A., Maggi,R. and Parnavelas,J. G. (2007) "From nose to fertility: the long migratory journey of gonadotropin-releasing hormone neurons." Trends Neurosci. **30**(12):638-644.

Cassidy,S. B., Schwartz,S., Miller,J. L. and Driscoll,D. J. (2012) "Prader-Willi syndrome." Genet.Med. **14**(1):10-26.

Castellano,J. M., Bentsen,A. H., Mikkelsen,J. D. and Tena-Sempere,M. (2010) "Kisspeptins: bridging energy homeostasis and reproduction." Brain Res. **1364**:129-138.

Chai,J. H., Locke,D. P., Greally,J. M., *et al.* (2003) "Identification of four highly conserved genes between breakpoint hotspots BP1 and BP2 of the Prader-Willi/Angelman syndromes deletion region that have undergone evolutionary transposition mediated by flanking duplicons." Am.J.Hum.Genet. **73**(4):898-925.

Chaillet,J. R., Knoll,J. H., Horsthemke,B. and Lalande,M. (1991) "The syntenic relationship between the critical deletion region for the Prader-Willi/Angelman syndromes and proximal mouse chromosome 7." Genomics **11**(3):773-776.

Challet,E., Pevet,P., Lakhdar-Ghazal,N. and Malan,A. (1997) "Ventromedial nuclei of the hypothalamus are involved in the phase advance of temperature and activity rhythms in food-restricted rats fed during daytime." Brain Res.Bull. **43**(2):209-218.

Chamberlain,S. J., Johnstone,K. A., DuBose,A. J., *et al.* (2004) "Evidence for genetic modifiers of postnatal lethality in PWS-IC deletion mice." Hum.Mol.Genet. **13**(23):2971-2977.

Chamberlain,S. J. and Brannan,C. I. (2001) "The Prader-Willi syndrome imprinting center activates the paternally expressed murine Ube3a antisense transcript but represses paternal Ube3a." Genomics **73**(3):316-322.

Choi,Y. H., Hartzell,D., Azain,M. J. and Baile,C. A. (2002) "TRH decreases food intake and increases water intake and body temperature in rats." Physiol.Behav. **77**(1):1-4.

Chou,T. C., Scammell,T. E., Gooley,J. J., Gaus,S. E., Saper,C. B. and Lu,J. (2003) "Critical role of dorsomedial hypothalamic nucleus in a wide range of behavioral circadian rhythms." J.Neurosci. **23**(33):10691-10702.

Clarke,D. J., Boer,H., Whittington,J., Holland,A., Butler,J. and Webb,T. (2002) "Prader-Willi syndrome, compulsive and ritualistic behaviours: the first population-based survey." Br.J.Psychiatry **180**:358-362.

Clarkson,J. and Herbison,A. E. (2006) "Development of GABA and glutamate signaling at the GnRH neuron in relation to puberty." Mol.Cell.Endocrinol. **254-255**:32-38.

Conejo,N. M., Gonzalez-Pardo,H., Lopez,M., Cantora,R. and Arias,J. L. (2007) "Induction of c-Fos expression in the mammillary bodies, anterior thalamus and dorsal hippocampus after fear conditioning." Brain Res.Bull. **74**(1-3):172-177.

Cowley,M. A., Smith,R. G., Diano,S., *et al.* (2003) "The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis." Neuron **37**(4):649-661.

Crino,A., Schiaffini,R., Ciampalini,P., *et al.* (2003) "Hypogonadism and pubertal development in Prader-Willi syndrome." Eur.J.Pediatr. **162**(5):327-333.

Cui,L. N., Saeb-Parsy,K. and Dyball,R. E. (1997) "Neurones in the supraoptic nucleus of the rat are regulated by a projection from the suprachiasmatic nucleus." J.Physiol. **502 (Pt 1)**(Pt 1):149-159.

Cummings,D. E. (2006) "Ghrelin and the short- and long-term regulation of appetite and body weight." Physiol.Behav. **89**(1):71-84.

Cummings,D. E., Clement,K., Purnell,J. Q., *et al.* (2002) "Elevated plasma ghrelin levels in Prader Willi syndrome." Nat.Med. **8**(7):643-644.

d'Anglemont de Tassigny,X., Fagg,L. A., Carlton,M. B. and Colledge,W. H. (2008) "Kisspeptin can stimulate gonadotropin-releasing hormone (GnRH) release by a direct action at GnRH nerve terminals." Endocrinology **149**(8):3926-3932.

de Smith,A. J., Purmann,C., Walters,R. G., *et al.* (2009) "A deletion of the HBII-85 class of small nucleolar RNAs (snoRNAs) is associated with hyperphagia, obesity and hypogonadism." Hum.Mol.Genet. **18**(17):3257-3265.

De Waele,K., Ishkanian,S. L., Bogarin,R., *et al.* (2008) "Long-acting octreotide treatment causes a sustained decrease in ghrelin concentrations but does not affect weight, behaviour and appetite in subjects with Prader-Willi syndrome." Eur.J.Endocrinol. **159**(4):381-388.

DelParigi,A., Tschop,M., Heiman,M. L., *et al.* (2002) "High circulating ghrelin: a potential cause for hyperphagia and obesity in prader-willi syndrome." J.Clin.Endocrinol.Metab. **87**(12):5461-5464.

Deponti,D., Francois,S., Baesso,S., *et al.* (2007) "Necdin mediates skeletal muscle regeneration by promoting myoblast survival and differentiation." J.Cell Biol. **179**(2):305-319.

Descheemaeker,M. J., Govers,V., Vermeulen,P. and Fryns,J. P. (2006) "Pervasive developmental disorders in Prader-Willi syndrome: the Leuven experience in 59 subjects and controls." Am.J.Med.Genet.A. **140**(11):1136-1142.

Ding,F., Li,H. H., Zhang,S., *et al.* (2008) "SnoRNA Snord116 (Pwcr1/MBII-85) deletion causes growth deficiency and hyperphagia in mice." PLoS One **3**(3):e1709.

Ding,F., Prints,Y., Dhar,M. S., *et al.* (2005) "Lack of Pwcr1/MBII-85 snoRNA is critical for neonatal lethality in Prader-Willi syndrome mouse models." Mamm.Genome **16**(6):424-431.

Doe,C. M., Relkovic,D., Garfield,A. S., *et al.* (2009) "Loss of the imprinted snoRNA mbii-52 leads to increased 5htr2c pre-RNA editing and altered 5HT2CR-mediated behaviour." Hum.Mol.Genet. **18**(12):2140-2148.

Down,J. L. (1887)"Mental Affections of Childhood and Youth "ChurchillLondon.

Downs,J. L. and Wise,P. M. (2009) "The role of the brain in female reproductive aging." Mol.Cell.Endocrinol. **299**(1):32-38.

Driscoll,D. J., Waters,M. F., Williams,C. A., *et al.* (1992) "A DNA methylation imprint, determined by the sex of the parent, distinguishes the Angelman and Prader-Willi syndromes." Genomics **13**(4):917-924.

Duker,A. L., Ballif,B. C., Bawle,E. V., *et al.* (2010) "Paternally inherited microdeletion at 15q11.2 confirms a significant role for the SNORD116 C/D box snoRNA cluster in Prader-Willi syndrome." Eur.J.Hum.Genet. **18**(11):1196-1201.

Dykens,E. and Shah,B. (2003) "Psychiatric disorders in Prader-Willi syndrome: epidemiology and management." CNS Drugs **17**(3):167-178.

Dykens,E. M., Leckman,J. F. and Cassidy,S. B. (1996) "Obsessions and compulsions in Prader-Willi syndrome." J.Child Psychol.Psychiatry **37**(8):995-1002.

Egawa,M., Inoue,S., Sato,S., Takamura,Y., Murakami,N. and Takahashi,K. (1991) "Restoration of circadian corticosterone rhythm in ventromedial hypothalamic lesioned rats." Neuroendocrinology **53**(6):543-548.

Eiholzer,U., Grieser,J., Schlumpf,M. and l'Allemand,D. (2007) "Clinical effects of treatment for hypogonadism in male adolescents with Prader-Labhart-Willi syndrome." Horm.Res. **68**(4):178-184.

Eldar-Geva,T., Hirsch,H. J., Benarroch,F., Rubinstein,O. and Gross-Tsur,V. (2010) "Hypogonadism in females with Prader-Willi syndrome from infancy to adulthood: variable combinations of a primary gonadal defect and hypothalamic dysfunction." Eur.J.Endocrinol. **162**(2):377-384.

Eldar-Geva,T., Hirsch,H. J., Rabinowitz,R., Benarroch,F., Rubinstein,O. and Gross-Tsur,V. (2009) "Primary ovarian dysfunction contributes to the hypogonadism in women with Prader-Willi Syndrome." Horm.Res. **72**(3):153-159.

Elias,C. F. (2012) "Leptin action in pubertal development: recent advances and unanswered questions." Trends Endocrinol.Metab. **23**(1):9-15.

Elias,C. F., Lee,C. E., Kelly,J. F., *et al.* (2001) "Characterization of CART neurons in the rat and human hypothalamus." J.Comp.Neurol. **432**(1):1-19.

Elias,C. F., Saper,C. B., Maratos-Flier,E., *et al.* (1998) "Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area." J.Comp.Neurol. **402**(4):442-459.

Erdie-Lalena,C. R., Holm,V. A., Kelly,P. C., Frayo,R. S. and Cummings,D. E. (2006) "Ghrelin levels in young children with Prader-Willi syndrome." J.Pediatr. **149**(2):199-204.

Fang,P., Lev-Lehman,E., Tsai,T. F., *et al.* (1999) "The spectrum of mutations in UBE3A causing Angelman syndrome." Hum.Mol.Genet. **8**(1):129-135.

Fang,P. H., Yu,M., Ma,Y. P., Li,J., Sui,Y. M. and Shi,M. Y. (2011) "Central nervous system regulation of food intake and energy expenditure: role of galanin-mediated feeding behavior." Neurosci.Bull. **27**(6):407-412.

Feigerlova,E., Diene,G., Conte-Auriol,F., *et al.* (2008) "Hyperghrelinemia precedes obesity in Prader-Willi syndrome." J.Clin.Endocrinol.Metab. **93**(7):2800-2805.

Fekete,C., Sarkar,S., Rand,W. M., *et al.* (2002) "Agouti-related protein (AGRP) has a central inhibitory action on the hypothalamic-pituitary-thyroid (HPT) axis; comparisons between the effect of AGRP and neuropeptide Y on energy homeostasis and the HPT axis." Endocrinology **143**(10):3846-3853.

Fekete,C., Kelly,J., Mihaly,E., *et al.* (2001) "Neuropeptide Y has a central inhibitory action on the hypothalamic-pituitary-thyroid axis." Endocrinology **142**(6):2606-2613.

Fekete,C., Legradi,G., Mihaly,E., *et al.* (2000a) "alpha-Melanocyte-stimulating hormone is contained in nerve terminals innervating thyrotropin-releasing hormone-synthesizing neurons in the hypothalamic paraventricular nucleus and prevents fasting-induced suppression of prothyrotropin-releasing hormone gene expression." J.Neurosci. **20**(4):1550-1558.

Fekete,C., Legradi,G., Mihaly,E., Tatro,J. B., Rand,W. M. and Lechan,R. M. (2000b) "alpha-Melanocyte stimulating hormone prevents fasting-induced suppression of corticotropin-releasing hormone gene expression in the rat hypothalamic paraventricular nucleus." Neurosci.Lett. **289**(2):152-156.

Fekete,C., Mihaly,E., Luo,L. G., *et al.* (2000c) "Association of cocaine- and amphetamine-regulated transcript-immunoreactive elements with thyrotropin-releasing hormone-synthesizing neurons in the hypothalamic paraventricular nucleus and its role in the regulation of the hypothalamic-pituitary-thyroid axis during fasting." J.Neurosci. **20**(24):9224-9234.

Feng,Z., Li,K., Liu,M. and Wen,C. (2010) "NRAGE is a negative regulator of nerve growth factor-stimulated neurite outgrowth in PC12 cells mediated through TrkA-ERK signaling." J.Neurosci.Res. **88**(8):1822-1828.

Fontes,M. A., Xavier,C. H., de Menezes,R. C. and Dimicco,J. A. (2011) "The dorsomedial hypothalamus and the central pathways involved in the cardiovascular response to emotional stress." Neuroscience **184**:64-74.

Freeman,M. E., Kanyicska,B., Lerant,A. and Nagy,G. (2000) "Prolactin: structure, function, and regulation of secretion." Physiol.Rev. **80**(4):1523-1631.

Fry,M. and Ferguson,A. V. (2010) "Ghrelin: central nervous system sites of action in regulation of energy balance." Int.J.Pept. **2010**:616757. Epub 2010 Feb 15.

Fry,M. and Ferguson,A. V. (2007) "The sensory circumventricular organs: brain targets for circulating signals controlling ingestive behavior." Physiol.Behav. **91**(4):413-423.

Fujiwara,K., Hasegawa,K., Ohkumo,T., Miyoshi,H., Tseng,Y. H. and Yoshikawa,K. (2012) "Necdin controls proliferation of white adipocyte progenitor cells." PLoS One **7**(1):e30948.

Gabriel,J. M., Merchant,M., Ohta,T., *et al.* (1999) "A transgene insertion creating a heritable chromosome deletion mouse model of Prader-Willi and angelman syndromes." Proc.Natl.Acad.Sci.U.S.A. **96**(16):9258-9263.

Gautron,L. and Elmquist,J. K. (2011) "Sixteen years and counting: an update on leptin in energy balance." J.Clin.Invest. **121**(6):2087-2093.

Gerard,M., Hernandez,L., Wevrick,R. and Stewart,C. L. (1999) "Disruption of the mouse necdin gene results in early post-natal lethality." Nat.Genet. **23**(2):199-202.

Glenn,C. C., Saitoh,S., Jong,M. T., *et al.* (1996) "Gene structure, DNA methylation, and imprinted expression of the human SNRPN gene." Am.J.Hum.Genet. **58**(2):335-346.

Glowa,J. R., Barrett,J. E., Russell,J. and Gold,P. W. (1992) "Effects of corticotropin releasing hormone on appetitive behaviors." Peptides **13**(3):609-621.

Goldstone,A. P. (2004) "Prader-Willi syndrome: advances in genetics, pathophysiology and treatment." Trends Endocrinol.Metab. **15**(1):12-20.

Goldstone,A. P., Thomas,E. L., Brynes,A. E., *et al.* (2004) "Elevated fasting plasma ghrelin in prader-willi syndrome adults is not solely explained by their reduced visceral adiposity and insulin resistance." J.Clin.Endocrinol.Metab. **89**(4):1718-1726.

Goldstone,A. P., Unmehopa,U. A. and Swaab,D. F. (2003) "Hypothalamic growth hormone-releasing hormone (GHRH) cell number is increased in human illness, but is not reduced in Prader-Willi syndrome or obesity." Clin.Endocrinol.(Oxf) **58**(6):743-755.

Goldstone,A. P., Unmehopa,U. A., Bloom,S. R. and Swaab,D. F. (2002) "Hypothalamic NPY and agouti-related protein are increased in human illness but not in Prader-Willi syndrome and other obese subjects." J.Clin.Endocrinol.Metab. **87**(2):927-937.

Goldstone,A. P., Thomas,E. L., Brynes,A. E., *et al.* (2001) "Visceral adipose tissue and metabolic complications of obesity are reduced in Prader-Willi syndrome female adults: evidence for novel influences on body fat distribution." J.Clin.Endocrinol.Metab. **86**(9):4330-4338.

Gooley,J. J., Schomer,A. and Saper,C. B. (2006) "The dorsomedial hypothalamic nucleus is critical for the expression of food-entrainable circadian rhythms." Nat.Neurosci. **9**(3):398-407.

Gray,T. A., Saitoh,S. and Nicholls,R. D. (1999) "An imprinted, mammalian bicistronic transcript encodes two independent proteins." Proc.Natl.Acad.Sci.U.S.A. **96**(10):5616-5621.

Greetfeld,M., Schmidt,M. V., Ganea,K., Sterlemann,V., Liebl,C. and Muller,M. B. (2009) "A single episode of restraint stress regulates central corticotrophin-releasing hormone receptor expression and binding in specific areas of the mouse brain." J.Neuroendocrinol. **21**(5):473-480.

Griffond,B. and Risold,P. Y. (2009) "MCH and feeding behavior-interaction with peptidic network." Peptides **30**(11):2045-2051.

Grugni,G., Crino,A., Bosio,L., *et al.* (2008) "The Italian National Survey for Prader-Willi syndrome: an epidemiologic study." Am.J.Med.Genet.A. **146**(7):861-872.

Gunay-Aygun,M., Schwartz,S., Heeger,S., O'Riordan,M. A. and Cassidy,S. B. (2001) "The changing purpose of Prader-Willi syndrome clinical diagnostic criteria and proposed revised criteria." Pediatrics **108**(5):E92.

Hahn,T. M., Breininger,J. F., Baskin,D. G. and Schwartz,M. W. (1998) "Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons." Nat.Neurosci. **1**(4):271-272.

Haqq,A. M., Farooqi,I. S., O'Rahilly,S., *et al.* (2003a) "Serum ghrelin levels are inversely correlated with body mass index, age, and insulin concentrations in normal children and are markedly increased in Prader-Willi syndrome." J.Clin.Endocrinol.Metab. **88**(1):174-178.

Haqq,A. M., Stadler,D. D., Rosenfeld,R. G., *et al.* (2003b) "Circulating ghrelin levels are suppressed by meals and octreotide therapy in children with Prader-Willi syndrome." J.Clin.Endocrinol.Metab. **88**(8):3573-3576.

Harding,S. M. and McGinnis,M. Y. (2005) "Microlesions of the ventromedial nucleus of the hypothalamus: effects on sociosexual behaviors in male rats." Behav.Neurosci. **119**(5):1227-1234.

Harding,S. M. and McGinnis,M. Y. (2003) "Effects of testosterone in the VMN on copulation, partner preference, and vocalizations in male rats." Horm.Behav. **43**(2):327-335.

Harris,G. C., Wimmer,M. and Aston-Jones,G. (2005) "A role for lateral hypothalamic orexin neurons in reward seeking." Nature **437**(7058):556-559.

Harvey,J. (2007) "Leptin regulation of neuronal excitability and cognitive function." Curr.Opin.Pharmacol. **7**(6):643-647.

Hasegawa,K. and Yoshikawa,K. (2008) "Necdin regulates p53 acetylation via Sirtuin1 to modulate DNA damage response in cortical neurons." J.Neurosci. **28**(35):8772-8784.

Hashimoto,T., Mori,K., Yoneda,Y., *et al.* (1998) "Proton magnetic resonance spectroscopy of the brain in patients with Prader-Willi syndrome." Pediatr.Neurol. **18**(1):30-35.

Hayashi,Y., Matsuyama,K., Takagi,K., Sugiura,H. and Yoshikawa,K. (1995) "Arrest of cell growth by necdin, a nuclear protein expressed in postmitotic neurons." Biochem.Biophys.Res.Commun. **213**(1):317-324.

Hill,J. W., Elmquist,J. K. and Elias,C. F. (2008) "Hypothalamic pathways linking energy balance and reproduction." Am.J.Physiol.Endocrinol.Metab. **294**(5):E827-32.

Hirsch,H. J., Eldar-Geva,T., Benarroch,F., Rubinstein,O. and Gross-Tsur,V. (2009) "Primary testicular dysfunction is a major contributor to abnormal pubertal development in males with Prader-Willi syndrome." J.Clin.Endocrinol.Metab. **94**(7):2262-2268.

Holley,C. L. and Topkara,V. K. (2011) "An introduction to small non-coding RNAs: miRNA and snoRNA." Cardiovasc.Drugs Ther. **25**(2):151-159.

Holm,V. A., Cassidy,S. B., Butler,M. G., *et al.* (1993) "Prader-Willi syndrome: consensus diagnostic criteria." Pediatrics **91**(2):398-402.

Hu,Y. and Bouloux,P. M. (2011) "X-linked GnRH deficiency: role of KAL-1 mutations in GnRH deficiency." Mol.Cell.Endocrinol. **346**(1-2):13-20.

Ibata,Y., Watanabe,K., Kinoshita,H., *et al.* (1979) "The location of LH-RH neurons in the rat hypothalamus and their pathways to the median eminence. Experimental immunohistochemistry and radioimmunoassay." Cell Tissue Res. **198**(3):381-395.

Ilnytska,O. and Argyropoulos,G. (2008) "The role of the Agouti-Related Protein in energy balance regulation." Cell Mol.Life Sci. **65**(17):2721-2731.

Israel,D. and Chua,S.,Jr. (2010) "Leptin receptor modulation of adiposity and fertility." Trends Endocrinol.Metab. **21**(1):10-16.

Iughetti,L., Bosio,L., Corrias,A., *et al.* (2008) "Pituitary height and neuroradiological alterations in patients with Prader-Labhart-Willi syndrome." Eur.J.Pediatr. **167**(6):701-702.

Jasoni,C. L., Porteous,R. W. and Herbison,A. E. (2009) "Anatomical location of mature GnRH neurons corresponds with their birthdate in the developing mouse." Dev.Dyn. **238**(3):524-531.

Jong,M. T., Carey,A. H., Caldwell,K. A., *et al.* (1999a) "Imprinting of a RING zinc-finger encoding gene in the mouse chromosome region homologous to the Prader-Willi syndrome genetic region." Hum.Mol.Genet. **8**(5):795-803.

Jong,M. T., Gray,T. A., Ji,Y., *et al.* (1999b) "A novel imprinted gene, encoding a RING zinc-finger protein, and overlapping antisense transcript in the Prader-Willi syndrome critical region." Hum.Mol.Genet. **8**(5):783-793.

Kageyama,H., Takenoya,F., Shiba,K. and Shioda,S. (2010) "Neuronal circuits involving ghrelin in the hypothalamus-mediated regulation of feeding." Neuropeptides **44**(2):133-138.

Kageyama,H., Kitamura,Y., Hosono,T., *et al.* (2008) "Visualization of ghrelin-producing neurons in the hypothalamic arcuate nucleus using ghrelin-EGFP transgenic mice." Regul.Pept. **145**(1-3):116-121.

Kanber,D., Giltay,J., Wieczorek,D., *et al.* (2009) "A paternal deletion of MKRN3, MAGEL2 and NDN does not result in Prader-Willi syndrome." Eur.J.Hum.Genet. **17**(5):582-590.

Kc,P., Balan,K. V., Tjoe,S. S., *et al.* (2010) "Increased vasopressin transmission from the paraventricular nucleus to the rostral medulla augments cardiorespiratory outflow in chronic intermittent hypoxia-conditioned rats." J.Physiol. **588**(Pt 4):725-740.

Keenan,D. M. and Veldhuis,J. D. (2009) "Age-dependent regression analysis of male gonadal axis." Am.J.Physiol.Regul.Integr.Comp.Physiol. **297**(5):R1215-27.

King,B. M. (2006) "The rise, fall, and resurrection of the ventromedial hypothalamus in the regulation of feeding behavior and body weight." Physiol.Behav. **87**(2):221-244.

King,J. C., Anthony,E. L., Fitzgerald,D. M. and Stopa,E. G. (1985) "Luteinizing hormone-releasing hormone neurons in human preoptic/hypothalamus: differential intraneuronal localization of immunoreactive forms." J.Clin.Endocrinol.Metab. **60**(1):88-97.

Kishino,T., Lalande,M. and Wagstaff,J. (1997) "UBE3A/E6-AP mutations cause Angelman syndrome." Nat.Genet. **15**(1):70-73.

Kishore,S., Khanna,A., Zhang,Z., *et al.* (2010) "The snoRNA MBII-52 (SNORD 115) is processed into smaller RNAs and regulates alternative splicing." Hum.Mol.Genet. **19**(7):1153-1164.

Kishore,S. and Stamm,S. (2006) "The snoRNA HBII-52 regulates alternative splicing of the serotonin receptor 2C." Science **311**(5758):230-232.

Knoll,J. H., Nicholls,R. D., Magenis,R. E., Graham,J. M.,Jr, Lalande,M. and Latt,S. A. (1989) "Angelman and Prader-Willi syndromes share a common chromosome 15 deletion but differ in parental origin of the deletion." Am.J.Med.Genet. **32**(2):285-290.

Kow,L. M. and Pfaff,D. W. (1991) "The effects of the TRH metabolite cyclo(His-Pro) and its analogs on feeding." Pharmacol.Biochem.Behav. **38**(2):359-364.

Kozlov,S. V., Bogenpohl,J. W., Howell,M. P., *et al.* (2007) "The imprinted gene Magel2 regulates normal circadian output." Nat.Genet. **39**(10):1266-1272.

Kubota,T., Li,N., Guan,Z., Brown,R. A. and Krueger,J. M. (2002) "Intrapreoptic microinjection of TNF-alpha enhances non-REM sleep in rats." Brain Res. **932**(1-2):37-44.

Kurita,M., Kuwajima,T., Nishimura,I. and Yoshikawa,K. (2006) "Necdin downregulates CDC2 expression to attenuate neuronal apoptosis." J.Neurosci. **26**(46):12003-12013.

Kuwajima,T., Hasegawa,K. and Yoshikawa,K. (2010) "Necdin promotes tangential migration of neocortical interneurons from basal forebrain." J.Neurosci. **30**(10):3709-3714.

Kuwajima,T., Nishimura,I. and Yoshikawa,K. (2006) "Necdin promotes GABAergic neuron differentiation in cooperation with Dlx homeodomain proteins." J.Neurosci. **26**(20):5383-5392.

Kuwajima,T., Taniura,H., Nishimura,I. and Yoshikawa,K. (2004) "Necdin interacts with the Msx2 homeodomain protein via MAGE-D1 to promote myogenic differentiation of C2C12 cells." J.Biol.Chem. **279**(39):40484-40493.

Kuwako,K., Hosokawa,A., Nishimura,I., *et al.* (2005) "Disruption of the paternal necdin gene diminishes TrkA signaling for sensory neuron survival." J.Neurosci. **25**(30):7090-7099.

Kuwako,K., Taniura,H. and Yoshikawa,K. (2004) "Necdin-related MAGE proteins differentially interact with the E2F1 transcription factor and the p75 neurotrophin receptor." J.Biol.Chem. **279**(3):1703-1712.

Kwon,I., Choe,H. K., Son,G. H. and Kim,K. (2011) "Mammalian molecular clocks." Exp.Neurobiol. **20**(1):18-28.

Lawrence,C. and Fraley,G. S. (2011) "Galanin-like peptide (GALP) is a hypothalamic regulator of energy homeostasis and reproduction." Front.Neuroendocrinol. **32**(1):1-9.

Lechan,R. M. and Fekete,C. (2006) "The TRH neuron: a hypothalamic integrator of energy metabolism." Prog.Brain Res. **153**:209-235.

Ledbetter,D. H., Riccardi,V. M., Airhart,S. D., Strobel,R. J., Keenan,B. S. and Crawford,J. D. (1981) "Deletions of chromosome 15 as a cause of the Prader-Willi syndrome." N.Engl.J.Med. **304**(6):325-329.

Lee,S., Walker,C. L., Karten,B., *et al.* (2005) "Essential role for the Prader-Willi syndrome protein necdin in axonal outgrowth." Hum.Mol.Genet. **14**(5):627-637.

Lee,S., Walker,C. L. and Wevrick,R. (2003) "Prader-Willi syndrome transcripts are expressed in phenotypically significant regions of the developing mouse brain." Gene Expr.Patterns **3**(5):599-609.

Lee,S., Kozlov,S., Hernandez,L., *et al.* (2000) "Expression and imprinting of MAGEL2 suggest a role in Prader-willi syndrome and the homologous murine imprinting phenotype." Hum.Mol.Genet. **9**(12):1813-1819.

LeFeuvre,R. A., Rothwell,N. J. and Stock,M. J. (1987) "Activation of brown fat thermogenesis in response to central injection of corticotropin releasing hormone in the rat." Neuropharmacology **26**(8):1217-1221.

Leibowitz,S. F. and Wortley,K. E. (2004) "Hypothalamic control of energy balance: different peptides, different functions." Peptides **25**(3):473-504.

Lein,E. S., Hawrylycz,M. J., Ao,N., *et al.* (2007) "Genome-wide atlas of gene expression in the adult mouse brain." Nature **445**(7124):168-176.

Leonard,C. M., Williams,C. A., Nicholls,R. D., *et al.* (1993) "Angelman and Prader-Willi syndrome: a magnetic resonance imaging study of differences in cerebral structure." Am.J.Med.Genet. **46**(1):26-33.

Madden,C. J. and Morrison,S. F. (2009) "Neurons in the paraventricular nucleus of the hypothalamus inhibit sympathetic outflow to brown adipose tissue." Am.J.Physiol.Regul.Integr.Comp.Physiol. **296**(3):R831-43.

Manning,L., Ohyama,K., Saeger,B., *et al.* (2006) "Regional morphogenesis in the hypothalamus: a BMP-Tbx2 pathway coordinates fate and proliferation through Shh downregulation." Dev.Cell. **11**(6):873-885.

Markakis,E. A. (2002) "Development of the neuroendocrine hypothalamus." Front.Neuroendocrinol. **23**(3):257-291.

Marsh,D. J., Weingarth,D. T., Novi,D. E., *et al.* (2002) "Melanin-concentrating hormone 1 receptor-deficient mice are lean, hyperactive, and hyperphagic and have altered metabolism." Proc.Natl.Acad.Sci.U.S.A. **99**(5):3240-3245.

Maruyama,K., Usami,M., Aizawa,T. and Yoshikawa,K. (1991) "A novel brain-specific mRNA encoding nuclear protein (necdin) expressed in neurally differentiated embryonal carcinoma cells." Biochem.Biophys.Res.Commun. **178**(1):291-296.

Mascari,M. J., Gottlieb,W., Rogan,P. K., *et al.* (1992) "The frequency of uniparental disomy in Prader-Willi syndrome. Implications for molecular diagnosis." N.Engl.J.Med. **326**(24):1599-1607.

Mathieu,J., Barth,A., Rosa,F. M., Wilson,S. W. and Peyrieras,N. (2002) "Distinct and cooperative roles for Nodal and Hedgehog signals during hypothalamic development." Development **129**(13):3055-3065.

Matsumoto,T. and Yamanouchi,K. (2000) "Acceleration of mounting behaviors in female rats by ibotenic acid lesions in the ventromedial hypothalamic nucleus." Neurosci.Lett. **291**(3):143-146.

Mauras,N., Rogol,A. D., Haymond,M. W. and Veldhuis,J. D. (1996) "Sex steroids, growth hormone, insulin-like growth factor-1: neuroendocrine and metabolic regulation in puberty." Horm.Res. **45**(1-2):74-80.

Melis,M. R. and Argiolas,A. (2011) "Central control of penile erection: a re-visitation of the role of oxytocin and its interaction with dopamine and glutamic acid in male rats." Neurosci.Biobehav.Rev. **35**(3):939-955.

Mercer,R. E., Chee,M. J. and Colmers,W. F. (2011) "The role of NPY in hypothalamic mediated food intake." Front.Neuroendocrinol. **32**(4):398-415.

Mercer,R. E., Kwolek,E. M., Bischof,J. M., van Eede,M., Henkelman,R. M. and Wevrick,R. (2009) "Regionally reduced brain volume, altered serotonin neurochemistry, and abnormal behavior in mice null for the circadian rhythm output gene Magel2." Am.J.Med.Genet.B.Neuropsychiatr.Genet. **150B**(8):1085-1099.

Mercer,R. E. and Wevrick,R. (2009) "Loss of magel2, a candidate gene for features of prader-willi syndrome, impairs reproductive function in mice." PLoS ONE **4**(1):e4291.

Messager,S., Chatzidaki,E. E., Ma,D., *et al.* (2005) "Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54." Proc.Natl.Acad.Sci.U.S.A. **102**(5):1761-1766.

Mieda,M., Williams,S. C., Richardson,J. A., Tanaka,K. and Yanagisawa,M. (2006) "The dorsomedial hypothalamic nucleus as a putative food-entrainable circadian pacemaker." Proc.Natl.Acad.Sci.U.S.A. **103**(32):12150-12155.

Millar,R. P. (2005) "GnRHs and GnRH receptors." Anim.Reprod.Sci. **88**(1-2):5-28.

Miller,J. L., Lynn,C. H., Driscoll,D. C., *et al.* (2011) "Nutritional phases in Prader-Willi syndrome." Am.J.Med.Genet.A. **155A**(5):1040-1049.

Miller,J. L., Goldstone,A. P., Couch,J. A., *et al.* (2008) "Pituitary abnormalities in Prader-Willi syndrome and early onset morbid obesity." Am.J.Med.Genet.A. **146A**(5):570-577.

Miller,J. L., Couch,J. A., Leonard,C. M., *et al.* (2007a) "Sylvian fissure morphology in Prader-Willi syndrome and early-onset morbid obesity." Genet.Med. **9**(8):536-543.

Miller,J. L., Couch,J. A., Schmalfuss,I., He,G., Liu,Y. and Driscoll,D. J. (2007b) "Intracranial abnormalities detected by three-dimensional magnetic resonance imaging in Prader-Willi syndrome." Am.J.Med.Genet.A. **143**(5):476-483.

Miller,N. L., Wevrick,R. and Mellon,P. L. (2009) "Necdin, a Prader-Willi syndrome candidate gene, regulates gonadotropin-releasing hormone neurons during development." Hum.Mol.Genet. **18**(2):248-260.

Milner,K. M., Craig,E. E., Thompson,R. J., *et al.* (2005) "Prader-Willi syndrome: intellectual abilities and behavioural features by genetic subtype." J.Child Psychol.Psychiatry **46**(10):1089-1096.

Miyakubo,H., Yamamoto,K., Hatakenaka,S., Hayashi,Y. and Tanaka,J. (2003) "Drinking decreases the noradrenaline release in the median preoptic area caused by hypovolemia in the rat." Behav.Brain Res. **145**(1-2):1-5.

Mochizuki,T., Arrigoni,E., Marcus,J. N., *et al.* (2011) "Orexin receptor 2 expression in the posterior hypothalamus rescues sleepiness in narcoleptic mice." Proc.Natl.Acad.Sci.U.S.A. **108**(11):4471-4476.

Mohawk,J. A., Green,C. B. and Takahashi,J. S. (2012) "Central and Peripheral Circadian Clocks in Mammals." Annu.Rev.Neurosci.

Moore,R. Y. and Eichler,V. B. (1972) "Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat." Brain Res. **42**(1):201-206.

Morton,G. J., Cummings,D. E., Baskin,D. G., Barsh,G. S. and Schwartz,M. W. (2006) "Central nervous system control of food intake and body weight." Nature **443**(7109):289-295.

Mouri,A., Sasaki,A., Watanabe,K., *et al.* (2012) "MAGE-D1 regulates expression of depression-like behavior through serotonin transporter ubiquitylation." J.Neurosci. **32**(13):4562-4580.

Murphy,K. G. and Bloom,S. R. (2006) "Gut hormones and the regulation of energy homeostasis." Nature **444**(7121):854-859.

Muscatelli,F., Abrous,D. N., Massacrier,A., *et al.* (2000) "Disruption of the mouse Necdin gene results in hypothalamic and behavioral alterations reminiscent of the human Prader-Willi syndrome." Hum.Mol.Genet. **9**(20):3101-3110.

Naik,D. V. (1975) "Immunoreactive LH-RH neurons in the hypothalamus identified by light and fluorescent microscopy." Cell Tissue Res. **157**(4):423-436.

Nicholls,R. D. and Knepper,J. L. (2001) "Genome organization, function, and imprinting in Prader-Willi and Angelman syndromes." Annu.Rev.Genomics Hum.Genet. **2**:153-175.

Nicholls,R. D., Knoll,J. H., Butler,M. G., Karam,S. and Lalande,M. (1989) "Genetic imprinting suggested by maternal heterodisomy in nondeletion Prader-Willi syndrome." Nature **342**(6247):281-285.

Niinobe,M., Koyama,K. and Yoshikawa,K. (2000) "Cellular and subcellular localization of necdin in fetal and adult mouse brain." Dev.Neurosci. **22**(4):310-319.

Nussey,S. and Whitehead,S. (2001) "Endocrinology: An Integrated Approach."

Onoe,H., Watanabe,Y., Ono,K., Koyama,Y. and Hayaishi,O. (1992) "Prostaglandin E2 exerts an awaking effect in the posterior hypothalamus at a site distinct from that mediating its febrile action in the anterior hypothalamus." J.Neurosci. **12**(7):2715-2725.

Osaka,T., Kawano,S., Ueta,Y., Inenaga,K., Kannan,H. and Yamashita,H. (1993) "Lateral preoptic neurons inhibit thirst in the rat." Brain Res.Bull. **31**(1-2):135-144.

Pagliardini,S., Ren,J., Wevrick,R. and Greer,J. J. (2005) "Developmental abnormalities of neuronal structure and function in prenatal mice lacking the prader-willi syndrome gene necdin." Am.J.Pathol. **167**(1):175-191.

Palermo,R. (2007) "Differential actions of FSH and LH during folliculogenesis." Reprod.Biomed.Online **15**(3):326-337.

Prader,A., Labhart,A. and Willi,H. (1956) "Ein syndrom von adipositas, kleinwuchs, kryptorchismus, and oligophrenie nach myatonieartigem zustand im neugeborenenalter." Schweizerische Medizinische Wochenschrift **86**:1260-1261.

Pralong,F. P., Gonzales,C., Voirol,M. J., *et al.* (2002) "The neuropeptide Y Y1 receptor regulates leptin-mediated control of energy homeostasis and reproductive functions." FASEB J. **16**(7):712-714.

Relkovic,D., Doe,C. M., Humby,T., *et al.* (2010) "Behavioural and cognitive abnormalities in an imprinting centre deletion mouse model for Prader-Willi syndrome." Eur.J.Neurosci. **31**(1):156-164.

Ren,J., Lee,S., Pagliardini,S., *et al.* (2003) "Absence of Ndn, encoding the Prader-Willi syndrome-deleted gene necdin, results in congenital deficiency of central respiratory drive in neonatal mice." J.Neurosci. **23**(5):1569-1573.

Roa,J., Garcia-Galiano,D., Varela,L., *et al.* (2009) "The mammalian target of rapamycin as novel central regulator of puberty onset via modulation of hypothalamic Kiss1 system." Endocrinology **150**(11):5016-5026.

Rogge,G., Jones,D., Hubert,G. W., Lin,Y. and Kuhar,M. J. (2008) "CART peptides: regulators of body weight, reward and other functions." Nat.Rev.Neurosci. **9**(10):747-758.

Runte,M., Huttenhofer,A., Gross,S., Kiefmann,M., Horsthemke,B. and Buiting,K. (2001) "The IC-SNURF-SNRPN transcript serves as a host for multiple small nucleolar RNA species and as an antisense RNA for UBE3A." Hum.Mol.Genet. **10**(23):2687-2700.

Sahoo,T., del Gaudio,D., German,J. R., *et al.* (2008) "Prader-Willi phenotype caused by paternal deficiency for the HBII-85 C/D box small nucleolar RNA cluster." Nat.Genet. **40**(6):719-721.

Sakurai,T. (2005) "Roles of orexin/hypocretin in regulation of sleep/wakefulness and energy homeostasis." Sleep Med.Rev. **9**(4):231-241.

Salehi,A. H., Roux,P. P., Kubu,C. J., *et al.* (2000) "NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and facilitates nerve growth factor-dependent apoptosis." Neuron **27**(2):279-288.

Sang,M., Wang,L., Ding,C., *et al.* (2011) "Melanoma-associated antigen genes - an update." Cancer Lett. **302**(2):85-90.

Schaller,F., Watrin,F., Sturny,R., Massacrier,A., Szepetowski,P. and Muscatelli,F. (2010) "A single postnatal injection of oxytocin rescues the lethal feeding behaviour in mouse newborns deficient for the imprinted Magel2 gene." Hum.Mol.Genet. **19**(24):4895-4905.

Schneider,J. E. (2004) "Energy balance and reproduction." Physiol.Behav. **81**(2):289-317.

Schulze,A., Mogensen,H., Hamborg-Petersen,B., Graem,N., Ostergaard,J. R. and Brondum-Nielsen,K. (2001) "Fertility in Prader-Willi syndrome: a case report with Angelman syndrome in the offspring." Acta Paediatr. **90**(4):455-459.

Schwanzel-Fukuda,M. and Pfaff,D. W. (1989) "Origin of luteinizing hormone-releasing hormone neurons." Nature **338**(6211):161-164.

Segal-Lieberman,G., Bradley,R. L., Kokkotou,E., *et al.* (2003) "Melanin-concentrating hormone is a critical mediator of the leptin-deficient phenotype." Proc.Natl.Acad.Sci.U.S.A. **100**(17):10085-10090.

Shioda,S., Kageyama,H., Takenoya,F. and Shiba,K. (2011) "Galanin-like peptide: a key player in the homeostatic regulation of feeding and energy metabolism?" Int.J.Obes.(Lond) **35**(5):619-628.

Sinnema,M., Boer,H., Collin,P., *et al.* (2011) "Psychiatric illness in a cohort of adults with Prader-Willi syndrome." Res.Dev.Disabil. **32**(5):1729-1735.

Skryabin,B. V., Gubar,L. V., Seeger,B., *et al.* (2007) "Deletion of the MBII-85 snoRNA gene cluster in mice results in postnatal growth retardation." PLoS Genet. **3**(12):e235.

Smith,J. T. (2011) "The role of kisspeptin and gonadotropin inhibitory hormone in the seasonal regulation of reproduction in sheep." Domest.Anim.Endocrinol.

Smith,M. S., True,C. and Grove,K. L. (2010) "The neuroendocrine basis of lactation-induced suppression of GnRH: role of kisspeptin and leptin." Brain Res. **1364**:139-152.

Sofikitis,N., Giotitsas,N., Tsounapi,P., Baltogiannis,D., Giannakis,D. and Pardalidis,N. (2008) "Hormonal regulation of spermatogenesis and spermiogenesis." J.Steroid Biochem.Mol.Biol. **109**(3-5):323-330.

Soni,S., Whittington,J., Holland,A. J., *et al.* (2008) "The phenomenology and diagnosis of psychiatric illness in people with Prader-Willi syndrome." Psychol.Med. **38**(10):1505-1514.

Soni,S., Whittington,J., Holland,A. J., *et al.* (2007) "The course and outcome of psychiatric illness in people with Prader-Willi syndrome: implications for management and treatment." J.Intellect.Disabil.Res. **51**(Pt 1):32-42.

State,M. W., Dykens,E. M., Rosner,B., Martin,A. and King,B. H. (1999) "Obsessive-compulsive symptoms in Prader-Willi and "Prader-Willi-Like" patients." J.Am.Acad.Child Adolesc.Psychiatry **38**(3):329-334.

Stephan,F. K. and Zucker,I. (1972) "Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions." Proc.Natl.Acad.Sci.U.S.A. **69**(6):1583-1586.

Suntsova,N., Guzman-Marin,R., Kumar,S., Alam,M. N., Szymusiak,R. and McGinty,D. (2007) "The median preoptic nucleus reciprocally modulates activity of arousal-related and sleep-related neurons in the perifornical lateral hypothalamus." J.Neurosci. **27**(7):1616-1630.

Sutcliffe,J. S., Nakao,M., Christian,S., *et al.* (1994) "Deletions of a differentially methylated CpG island at the SNRPN gene define a putative imprinting control region." Nat.Genet. **8**(1):52-58.

Suter,K. J., Pohl,C. R. and Wilson,M. E. (2000) "Circulating concentrations of nocturnal leptin, growth hormone, and insulin-like growth factor-I increase before the onset of puberty in agonadal male monkeys: potential signals for the initiation of puberty." J.Clin.Endocrinol.Metab. **85**(2):808-814.

Swaab,D. F., Purba,J. S. and Hofman,M. A. (1995) "Alterations in the hypothalamic paraventricular nucleus and its oxytocin neurons (putative satiety cells) in Prader-Willi syndrome: a study of five cases." J.Clin.Endocrinol.Metab. **80**(2):573-579.

Szabo,N. E., Zhao,T., Cankaya,M., Theil,T., Zhou,X. and Alvarez-Bolado,G. (2009) "Role of neuroepithelial Sonic hedgehog in hypothalamic patterning." J.Neurosci. **29**(21):6989-7002.

Szarek,E., Cheah,P. S., Schwartz,J. and Thomas,P. (2010) "Molecular genetics of the developing neuroendocrine hypothalamus." Mol.Cell.Endocrinol. **323**(1):115-123.

Szawka,R. E., Ribeiro,A. B., Leite,C. M., *et al.* (2010) "Kisspeptin regulates prolactin release through hypothalamic dopaminergic neurons." Endocrinology **151**(7):3247-3257.

Takazaki,R., Nishimura,I. and Yoshikawa,K. (2002) "Necdin is required for terminal differentiation and survival of primary dorsal root ganglion neurons." Exp.Cell Res. **277**(2):220-232.

Tan,T. M., Vanderpump,M., Khoo,B., Patterson,M., Ghatei,M. A. and Goldstone,A. P. (2004) "Somatostatin infusion lowers plasma ghrelin without reducing appetite in adults with Prader-Willi syndrome." J.Clin.Endocrinol.Metab. **89**(8):4162-4165.

Taniura,H., Kobayashi,M. and Yoshikawa,K. (2005) "Functional domains of necdin for protein-protein interaction, nuclear matrix targeting, and cell growth suppression." J.Cell.Biochem. **94**(4):804-815.

Taniura,H., Matsumoto,K. and Yoshikawa,K. (1999) "Physical and functional interactions of neuronal growth suppressor necdin with p53." J.Biol.Chem. **274**(23):16242-16248.

Tennese,A. A., Gee,C. B. and Wevrick,R. (2008) "Loss of the Prader-Willi syndrome protein necdin causes defective migration, axonal outgrowth, and survival of embryonic sympathetic neurons." Dev.Dyn. **237**(7):1935-1943.

Thornhill,J. A. and Halvorson,I. (1994) "Electrical stimulation of the posterior and ventromedial hypothalamic nuclei causes specific activation of shivering and nonshivering thermogenesis." Can.J.Physiol.Pharmacol. **72**(1):89-96.

Toni,R., Malaguti,A., Benfenati,F. and Martini,L. (2004) "The human hypothalamus: a morpho-functional perspective." J.Endocrinol.Invest. **27**(6 Suppl):73-94.

Tsai,T. F., Armstrong,D. and Beaudet,A. L. (1999) "Necdin-deficient mice do not show lethality or the obesity and infertility of Prader-Willi syndrome." Nat.Genet. **22**(1):15-16.

Tsujino,N. and Sakurai,T. (2009) "Orexin/hypocretin: a neuropeptide at the interface of sleep, energy homeostasis, and reward system." Pharmacol.Rev. **61**(2):162-176.

Uehara,Y., Shimizu,H., Ohtani,K., Sato,N. and Mori,M. (1998) "Hypothalamic corticotropin-releasing hormone is a mediator of the anorexigenic effect of leptin." Diabetes **47**(6):890-893.

Vadakkadath Meethal,S. and Atwood,C. S. (2005) "The role of hypothalamic-pituitary-gonadal hormones in the normal structure and functioning of the brain." Cell Mol.Life Sci. **62**(3):257-270.

Vann,S. D. and Aggleton,J. P. (2004) "The mammillary bodies: two memory systems in one?" Nat.Rev.Neurosci. **5**(1):35-44.

Veldhuis,J. D., Keenan,D. M., Liu,P. Y., Iranmanesh,A., Takahashi,P. Y. and Nehra,A. X. (2009) "The aging male hypothalamic-pituitary-gonadal axis: pulsatility and feedback." Mol.Cell.Endocrinol. **299**(1):14-22.

Veltman,M. W., Thompson,R. J., Roberts,S. E., Thomas,N. S., Whittington,J. and Bolton,P. F. (2004) "Prader-Willi syndrome--a study comparing deletion and uniparental disomy cases with reference to autism spectrum disorders." Eur.Child Adolesc.Psychiatry **13**(1):42-50.

Wang,X., Tang,J., Xing,L., *et al.* (2010) "Interaction of MAGED1 with nuclear receptors affects circadian clock function." EMBO J. **29**(8):1389-1400.

Wawrzik,M., Unmehopa,U. A., Swaab,D. F., van de Nes,J., Buiting,K. and Horsthemke,B. (2010) "The C15orf2 gene in the Prader-Willi syndrome region is subject to genomic imprinting and positive selection." Neurogenetics **11**(2):153-161.

Weaver,D. R. (1998) "The suprachiasmatic nucleus: a 25-year retrospective." J.Biol.Rhythms **13**(2):100-112.

Wigren,M. and Hansen,S. (2003) "Rituals and compulsivity in Prader-Willi syndrome: profile and stability." J.Intellect.Disabil.Res. **47**(Pt 6):428-438.

Willie,J. T., Chemelli,R. M., Sinton,C. M. and Yanagisawa,M. (2001) "To eat or to sleep? Orexin in the regulation of feeding and wakefulness." Annu.Rev.Neurosci. **24**:429-458.

Wintermantel,T. M., Campbell,R. E., Porteous,R., *et al.* (2006) "Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility." Neuron **52**(2):271-280.

Wray,S. (2001) "Development of luteinizing hormone releasing hormone neurones." J.Neuroendocrinol. **13**(1):3-11.

Wray,S., Key,S., Qualls,R. and Fueshko,S. M. (1994) "A subset of peripherin positive olfactory axons delineates the luteinizing hormone releasing hormone neuronal migratory pathway in developing mouse." Dev.Biol. **166**(1):349-354.

Wray,S., Grant,P. and Gainer,H. (1989) "Evidence that cells expressing luteinizing hormone-releasing hormone mRNA in the mouse are derived from progenitor cells in the olfactory placode." Proc.Natl.Acad.Sci.U.S.A. **86**(20):8132-8136.

Wyss,J. M., Tilokskulchai,K., King,K., Kadish,I. and van Groen,T. (1999) "Distribution of neurons in the anterior hypothalamic nucleus activated by blood pressure changes in the rat." Brain Res.Bull. **49**(3):163-172.

Wyss,J. M., Yang,R. H. and Oparil,S. (1990) "Lesions of the anterior hypothalamic area increase arterial pressure in NaCl-sensitive spontaneously hypertensive rats." J.Auton.Nerv.Syst. **31**(1):21-29.

Xu,Y., Elmquist,J. K. and Fukuda,M. (2011) "Central nervous control of energy and glucose balance: focus on the central melanocortin system." Ann.N.Y.Acad.Sci. **1243**:1-14.

Yang,T., Adamson,T. E., Resnick,J. L., *et al.* (1998) "A mouse model for Prader-Willi syndrome imprinting-centre mutations." Nat.Genet. **19**(1):25-31.

Yoshida,K., Tobet,S. A., Crandall,J. E., Jimenez,T. P. and Schwarting,G. A. (1995) "The migration of luteinizing hormone-releasing hormone neurons in the developing rat is associated with a transient, caudal projection of the vomeronasal nerve." J.Neurosci. **15**(12):7769-7777.

Zhang,W., Wang,D., Liu,X. H., Kosala,W. R., Rajapaksha,J. S. and Fisher,T. E. (2009) "An osmosensitive voltage-gated K+ current in rat supraoptic neurons." Eur.J.Neurosci. **29**(12):2335-2346.

Zhang,W., Star,B., Rajapaksha,W. R. and Fisher,T. E. (2007) "Dehydration increases L-type Ca(2+) current in rat supraoptic neurons." J.Physiol. **580**(Pt 1):181-193.

Zorrilla,E. P., Tache,Y. and Koob,G. F. (2003) "Nibbling at CRF receptor control of feeding and gastrocolonic motility." Trends Pharmacol.Sci. **24**(8):421-427.

Chapter 2. Regionally reduced brain volume, altered serotonin neurochemistry, and abnormal behaviour in mice null for the circadian rhythm output gene *Magel2*.

This chapter is a modified version of a previously published manuscript

Mercer, R.E., Kwolek, E.M., Bischof, J.M., van Eede, M., Henkelman, R.M., and Wevrick, R. (2009) Regionally reduced brain volume, altered serotonin neurochemistry, and abnormal behaviour in mice null for the circadian rhythm output gene *Magel2*. *Am J Med Genet B Neuropsychiatr Genet* 150B(8):1085-1099.

R.E. Mercer completed examination of developmental milestones, examined numbers and locations of brainstem serotonin neurons and hypothalamic dopamine neurons, completed the marble burying, novel object, and grooming studies, and assisted with manuscript preparation. Remaining behavioural tests were performed at the Centre for Modeling Human Disease in Toronto, Ontario, and were analyzed by J.M. Bischof, who also assisted with manuscript preparation. E.M. Kwolek prepared brain samples for neurochemical analysis, and assisted with the marble burying study. M. van Eede and R.M. Henkelman performed and analyzed the MRI experiments, and assisted with manuscript preparation.

2.1 Introduction

MAGEL2 is a member of the Type II melanoma-associated antigen gene (MAGE) protein family, which share a protein–protein interaction domain called the MAGE homology domain (Chomez *et al.*, 2001, Barker and Salehi, 2002). *Magel2* is expressed in the developing murine central nervous system and in cell lines that model neuronal differentiation (Boccaccio *et al.*, 1999, Lee *et al.*, 2000). *Magel2* expression is apparent in the hypothalamus, the control center in the brain for endocrine function, circadian rhythm, appetite, thirst, and thermoregulation (Moore *et al.*, 2002), with maximal levels in the suprachiasmatic nucleus (a region of the anterior ventral hypothalamus) and the arcuate nucleus (Lee *et al.*, 2000, Lee *et al.*, 2003b).

MAGEL2 and *NDN*, the gene that encodes the MAGE protein NECDIN, are among a small set of genes that are typically inactivated in Prader–Willi syndrome (PWS). PWS is a congenital disorder characterized by symptoms of varying severity among affected individuals: intellectual disability, hypotonia, short stature, childhood-onset hyperphagia often leading to obesity, excessive sleepiness, neuroendocrine abnormalities, and incomplete sexual development (Gunay-Aygun *et al.*, 2001, Eiholzer and Whitman, 2004, Goldstone., 2004). While physiological abnormalities in PWS point to a defect in the development or function of the hypothalamus, the typical behavioural profile suggests deficits in other parts of the brain also contribute to this complex disorder.

The Wevrick laboratory has previously described a mouse strain carrying a gene-targeted lacZ insertion into the *Magel2* locus, creating a null *Magel2* allele (Bischof *et al.*, 2007, Kozlov *et al.*, 2007). *Magel2*-null mice display defective

maintenance of their circadian rhythm, reduced orexin in the lateral hypothalamus (Kozlov *et al.*, 2007), and reproductive deficits (Mercer and Wevrick, 2009), reflecting a phenotype that is consistent with a hypothalamic defect. They also have substantially reduced total activity measured by 24-hr wheel running but only slightly reduced food intake. This excessive food intake for their reduced energy output eventually leads to significantly increased adiposity and a correspondingly altered metabolic profile (Bischof *et al.*, 2007). To explore a possible role for the *Magel2* gene in learning and behaviour, *Magel2*-null mice were subjected to a series of standardized behavioural tests. A high-resolution magnetic resonance imaging (MRI) study of the brain was performed, and brain tissue was analyzed for altered levels of biogenic amines. We now report that loss of *Magel2* affects murine behaviour and neurochemistry, and reduces brain volume, particularly in areas involved in reward, emotion, and memory.

2.2 Materials and Methods

Magel2-null mice

All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the local Animal Policy and Welfare Committees. The *Magel2* mouse colony is maintained on a C57Bl/6 background by breeding heterozygous *Magel2*-m/+p mice carrying a maternally inherited *Magel2-lacZ* knock-in allele with C57Bl/6 male mice to generate heterozygous, functionally wildtype offspring. Heterozygous carrier males were then bred with wildtype females to produce experimental animals. This cross generated *Magel2*+m/-p mice that inherited the gene-targeted allele

from their fathers, and which are *Magel2*-null because they lack expression of *Magel2* due to genomic imprinting that silences the maternally inherited allele. *Magel2*+/+ littermates serve as controls. Identification of *Magel2*-null offspring was determined by PCR genotyping of DNA extracted by ear notch biopsy using lacZ oligonucleotide primers (LACZ754F 5’ CGT GAC TAC CTA CGG GTA AC; and LACZ1153R 5’ AGT TGT TCT GCT TCA TCA GC), which will only produce a band in mice carrying the targeted *Magel2* allele. As a genomic control, Dlxin oligonucleotide primers (DLXIN1F 5’ CCT TGC TTG TGC AGA CCT TG; and DLXIN2R 5’ GGC AGC ATG TGG ACC TTT AG) were used, producing a band in all mice with successful DNA extraction.

Four to eight mice of each genotype at embryonic day 18 and seven male mice of each genotype at 20–24 weeks of age were used for neurochemical profiling. Unless otherwise noted, behaviour tests were performed on adult male *Magel2*-null mice and wild-type control littermates, $n = 5–6$ of each genotype, aged 14–26 weeks, and housed under 12 hr on, 12 hr off lighting at the Centre for Modeling Human Disease in Toronto, Canada. Fat mass, lean mass, and bone mineral content were measured using a PIXI-mus instrument. These same male mice were MRI imaged at 26 weeks of age. The marble burying, novel object exploration, and grooming tests were performed on male and female age-matched adult mice ($n = 12–20$ of each sex and genotype) in the animal housing unit at the University of Alberta.

Sample preparation for MRI

Magel2-null mice (n = 6) and their wild-type littermates (n = 6) were anesthetized at 26 weeks of age with a combination of Ketamine (100 mg/kg, Pfizer, Kirkland, QC, Canada) and Rompun (20 mg/kg, Bayer, Inc., Toronto, ON, Canada) via intraperitoneal injection. A previously described sample preparation protocol for scanning was used with slight modifications (Tyszka *et al.*, 2006). Thoracic cavities were exposed, and the mice were perfused through the left ventricle with 30 ml of phosphate-buffered saline (PBS, pH 7.4) at room temperature (25°C) at a rate of approximately 1 ml/min. This was followed by infusion with 30 ml of iced 4% paraformaldehyde in PBS. Following perfusion, the heads were removed along with the skin, lower jaw, ears, and the cartilaginous nose tip. The remaining skull structures were post-fixed in 4% paraformaldehyde at 4°C for 12 hr. Following an incubation period of 5 days in PBS and 0.01% sodium azide at 15°C, the skulls were transferred to a PBS and 2 mM ProHance® (gadoteridol, Bracco Diagnostics, Inc., Princeton, NJ) contrast agent solution for at least 7 days at 15°C.

MRI image acquisition

A multi-channel 7.0-T MRI scanner (Varian, Inc., Palo Alto, CA) with a 6 cm inner bore diameter insert gradient set was used to acquire anatomical images of brains within skulls. Prior to imaging, the samples were removed from the contrast agent solution, blotted and placed into 13 mm diameter plastic tubes filled with a proton-free susceptibility-matching fluid (Fluorinert FC-77, 3 M Corp., St. Paul, MN). Three custom-built, 14 mm diameter solenoid coils with a

length of 18.3 mm and over wound ends were used to image three brains in parallel. Parameters used in the scans were optimized for gray/white matter contrast: a T2-weighted, 3D fast spin-echo sequence, with TR/TE = 325/32 msec, four averages, field-of-view 14 mm × 14 mm × 25 mm and matrix size = 432 × 432 × 780 giving an image with 32 μm isotropic voxels. Total imaging time was 11.3 hr (Henkelman *et al.*, 2006).

Image processing

The 32 μm isotropic resolution T2-weighted MRI scans were nonlinearly aligned to a three dimensional atlas of the mouse brain with 62 structures identified (Dorr *et al.*, 2008). This process consisted of an initial step in which all of the MRI scans were nonlinearly aligned to each other using an unbiased group wise registration algorithm (Kovacevic *et al.*, 2005). Briefly, rigid body registration was carried out towards a pre-existing image based on the same mouse strain as reported previously (Collins *et al.*, 1994). All possible pair-wise 12-parameter registrations were then carried out to create an unbiased linear average model of the entire data set. All images were subsequently nonlinearly aligned towards the 12-parameter average. The resulting registered MRIs were resampled and averaged (Kovacevic *et al.*, 2005, Collins *et al.*, 1994). This iterative procedure was repeated for an additional five generations with increasingly fine deformation grid-point spacings. The end result was that all 12 scans were deformed into exact alignment with each other in an unbiased fashion. This allowed for the analysis of the deformations needed to take each mouse's anatomy into this final atlas space, the goal being to model how the deformation

fields relate to genotype. Correspondence with the 3D atlas was obtained by nonlinear alignment of the final stage average MRI with the 40-mouse average MRI upon which the atlas is based (Dorr *et al.*, 2008).

MRI analysis

Local differences in brain shape related to genotype were assessed by analysis of the deformation fields (Gaser *et al.*, 1999, Nieman *et al.*, 2006). To reduce random noise and assure normality under the central limit theorem, the transformation data were blurred prior to analysis with a Gaussian kernel with a full width at half maximum of 1 mm, and the logarithm of the Jacobian was computed for univariate statistical comparison at every image point. This statistical analysis results in millions of separate statistical tests. In order to account for an inflated type I error, the False Discovery Rate (FDR) technique was applied (Genovese *et al.*, 2002) with a 10% FDR threshold. The threshold corresponded to an uncorrected P-value of 0.00031. The interpretation of these results is that, on average, 10% of the voxels shown as significant will be false positives. The volume for each anatomical structure defined in the atlas was computed for each mouse by integrating the Jacobian of the transformation mapping the atlas image to the image for that mouse. This procedure has previously been shown to provide volume estimates comparable to those obtained by standard stereological methods using tissue sections (Lerch *et al.*, 2008a).

Neurochemical analysis

Brain regions were dissected from embryonic or adult *Magel2*-null or wild-type littermate control mice, snap frozen on dry ice, then stored at −80°C.

Brain samples were processed for HPLC combined with fluorescence detection to measure levels of biogenic amines (noradrenaline (NA), dihydroxyphenylacetic acid (DOPAC), dopamine (DA), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), serotonin (5-HT) and amino acids (Trp, Asp, Glu, Asn, Ser, Gln, Gly, Taur, Ala, GABA)) as described (Parent *et al.*, 2001). Two-way ANOVA with Bonferroni post-test analysis was performed to analyze the effect of genotype on measures of dopamine and serotonin pathways, using GraphPad Prism (San Diego, CA). Immunohistochemistry, confocal microscopy imaging, and cell counts of mouse brain sections was performed using a primary antibody directed against tyrosine hydroxylase (TH; Chemicon (Millipore) Billerica, MA) or against 5-HT (Immunostar, Hudson, WI) as previously described (Pagliardini *et al.*, 2005).

Behavioural analysis

Male *Magel2*-null mice and their control littermates were tested in the following order at the Centre for Modeling Human Disease: modified SHIRPA for general health, appearance, and neurological reflexes (Rogers *et al.*, 1997), open field screen, acoustic startle/pre-pulse inhibition screen, tail suspension test, 1-day Rotarod test, learning and memory screen, elevated plus maze, beam test, and 3-day Rotarod test. Before each test, the mice were acclimatized to the testing room for at least 30 min. Test rooms were purpose-designed for behaviour testing and controlled for noise, light, humidity, and ventilation. The same observer performed all tests at the Centre for Modeling Human Disease and was blinded with respect to the genotype of the mice. In the open field screen, rearing, time

spent in the border area, and time spent in the center area were recorded during the first 5 min. Horizontal and vertical activities were recorded in 5 min intervals for a total of 30 min. The acoustic startle and pre-pulse inhibition screen is generally used to assess sensorimotor gating deficits and was performed as described (Clapcote *et al.*, 2007). The tail suspension test of depressive traits, the 1- and 3-day Rotarod screen, and the elevated plus maze were performed as described (Soleimani *et al.*, 2008).

The contextual and cued fear conditioning test was performed using the Video Fear Conditioning system (Med Associates, St. Albans, VT) essentially as described (Clapcote *et al.*, 2005). The apparatus applied foot shocks and auditory tones while recording the activity within the chamber, specifically noting freezing time during the segments of the procedure. Each mouse was acclimatized to the test chamber for 2 min. without any stimulus, to record baseline activity. A 30 sec auditory tone was then applied, accompanied by a foot shock in the last 2 sec of the tone (the conditioning stimulus). Following the shock, the mouse stayed in the chamber for 30 sec before removal to the home cage. The test of contextual memory was performed approximately 24 hr after the conditioning session. The mouse was returned to the test context chamber and freezing behaviour measured over 5 min. without any stimulus. The mouse was then placed back into the home cage and allowed to rest for 2 hr before the cued memory test was performed. During this time, the environment within the testing chamber was changed to provide an altered context. The grid floor was covered with a plastic sheet, two pieces of fiberglass were used to create a triangle-shaped chamber, the door was

covered with black cardboard and 1% acetic acid was used to wipe down the surfaces. The mouse was placed in the altered context chamber and acclimatized for 3 min. without any stimulus to obtain a Day 2 pre-tone baseline measurement of freezing. This was followed by the application of a 3 min. auditory tone, during which time an additional freezing measurement was made. The mouse was then placed back into its home cage.

The custom apparatus for the beam test is made up of two platforms (25 cm × 35 cm) that are connected with a 90 cm long round beam (18 mm diameter) suspended 50 cm above the floor. One platform was brightly illuminated while the opposite platform was dark and contained a box providing an enclosed safety area for the mouse. Four consecutive training trials to assist the mouse in navigating the beam were performed on the first day. On the second day, a single trial that recorded the latency to traverse the beam and the number of times the hind feet slipped off was performed.

The one-trial object recognition task (Hammond *et al.*, 2004) was performed in empty rat test cages (26 cm × 48 cm × 20 cm) in which the mouse was habituated for 15 min. Two identical “sample” objects (e.g., a 15 ml plastic tube, a scintillation fluid vial, a syringe barrel) were placed at opposite ends of the cage, ensuring enough room on all sides of the objects for the mouse to walk. The exploration time of the sample objects, defined as the head oriented towards object, 2 cm away or closer, was recorded. The mouse was allowed to explore the sample objects until a total of 38 sec exploration had occurred. The mouse was returned to its home cage for a 5 min recovery period, during which time the test

cage was cleaned with 10% ethanol and a familiar sample object and a new “test” object were placed in the test cage. The mouse was returned to the test cage, and the time spent with each object over a 5 min period was recorded. On Day 2, a “sample” object (used in previous day's test), and a new “test” object were placed in the test cage. The mouse was introduced to the test environment, and time spent with each object over a 5 min period was again recorded. Control mice typically spend more time exploring the test object over a retention time of minutes to days, suggesting that they recognize the familiar object. For the marble burying analysis, mice were tested individually in clear mouse cages with 5 cm of bedding, in the dark. Each mouse was allowed a minimum of 2 hr to acclimate to the testing room and cage, after which the mice were removed while testers placed twelve clear glass marbles (0.6 cm in diameter—three rows of four) in each cage. Mice were returned to the testing cage and were removed to their home cages after 3 min. The number of marbles that were not buried was recorded. Marbles were considered buried if covered at least 75% with bedding (Deacon., 2006). The grooming test was performed in a clean mouse cage with standard bedding. The grooming pattern (direction, length of time) was observed when the mouse was placed in the new cage, and after a gentle misting with water (Kalueff *et al.*, 2007).

Behavioural data were analyzed statistically by t-test except the horizontal and vertical activity profile, the 3-day Rotarod test, and the fear-conditioning test, which were analyzed by two-way ANOVA with Bonferroni post-tests, using GraphPad Prism. Probabilities <0.05 were deemed significant.

2.3 Results

2.3.1 Expression of Magel2 in the adult mouse brain

Expression of *Magel2* in the embryonic brain by RNA in situ hybridization was previously outlined (Lee *et al.*, 2000, Lee *et al.*, 2003a), and described expression in the hypothalamus, lateral septal complex, and the bed nucleus of the stria terminalis by staining for the lacZ reporter gene in brain from *Magel2*-null adult mice (Kozlov *et al.*, 2007). To refine the regions of *Magel2* expression, we examined the Allen Brain Atlas, an on-line resource that catalogues RNA in situ hybridization profiles of the adult mouse brain (Lein *et al.*, 2007). Highest levels of expression were observed in the hypothalamus, with expression also seen in the medial septal nucleus, lateral septal complex and bed nucleus of the stria terminalis. Single cells in the outer region of the external granule layer of the cerebellum also express *Magel2*, and expression was present broadly throughout the pons and the medulla, and in the nucleus of the solitary tract (Figure 2.1).

2.3.2 General behaviour and health of Magel2-null mice

No overt differences in the acquisition of developmental milestones (e.g., physical development, rooting reflex, righting reflex, forelimb/hind limb grasping, or locomotor behaviour (Crawley., 2007) were noted in *Magel2*-null mice compared to control littermates from birth to 3 weeks of age, suggesting adequate muscle tone (see P7 example, Table 2.1). There was a 5–10% reduction in pre-weaning body weight similar to that previously reported (Bischof *et al.*, 2007). All adult mice were also healthy, and *Magel2*-null mice performed normally in a

formal screen for general health and neurological reflexes (SHIRPA screen (Rogers *et al.*, 1997)). Adult body composition abnormalities were consistent with our previous report, with increased fat mass (131% of control, $P < 0.01$) and decreased lean mass (92% of control, $P < 0.005$), measured by a PIXI-mus small animal densitometer at 26 weeks. Decreased bone mineral content (87% of control, $P < 0.05$) was present, but total body mass was not significantly different between genotypes. Gait and postural abnormalities were occasionally observed in the home cages, with *Magel2*-null mice displaying elevated head position during ambulation and hopping movements with both hind limbs together, movements that were never observed in wild-type mice.

Table 2.1 Developmental milestones observed at postnatal day 7 (P7)

	Weight (g)	Brain Weight (g)	Body Length (cm)	Righting Reflex (s)	Negative Geotaxis (s)	Forelimb Grasp	Hindlimb Grasp
Control							
(n=8)	4.02 ± 0.08	0.23 ± 0.01	4.1 ± 0.1	1.8 ± 0.8	11.1 ± 2.6	8/8	5/8
***Magel2* -null**							
(n=7)	3.61 ± 0.07	0.23 ± 0.01	3.9 ± 0.1	2.5 ± 1.2	11.4 ± 2.4	7/7	3/7
p Value	0.004	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Righting reflex measures the time in seconds it takes for a pup to roll over when placed on their back. Negative geotaxis measures the time in seconds for a pup placed on a downward angle (30 °) to rotate to the upwards facing direction. Grasping tests examined ability to grasp and hold a narrow diameter plastic rod against withdrawl pressure.

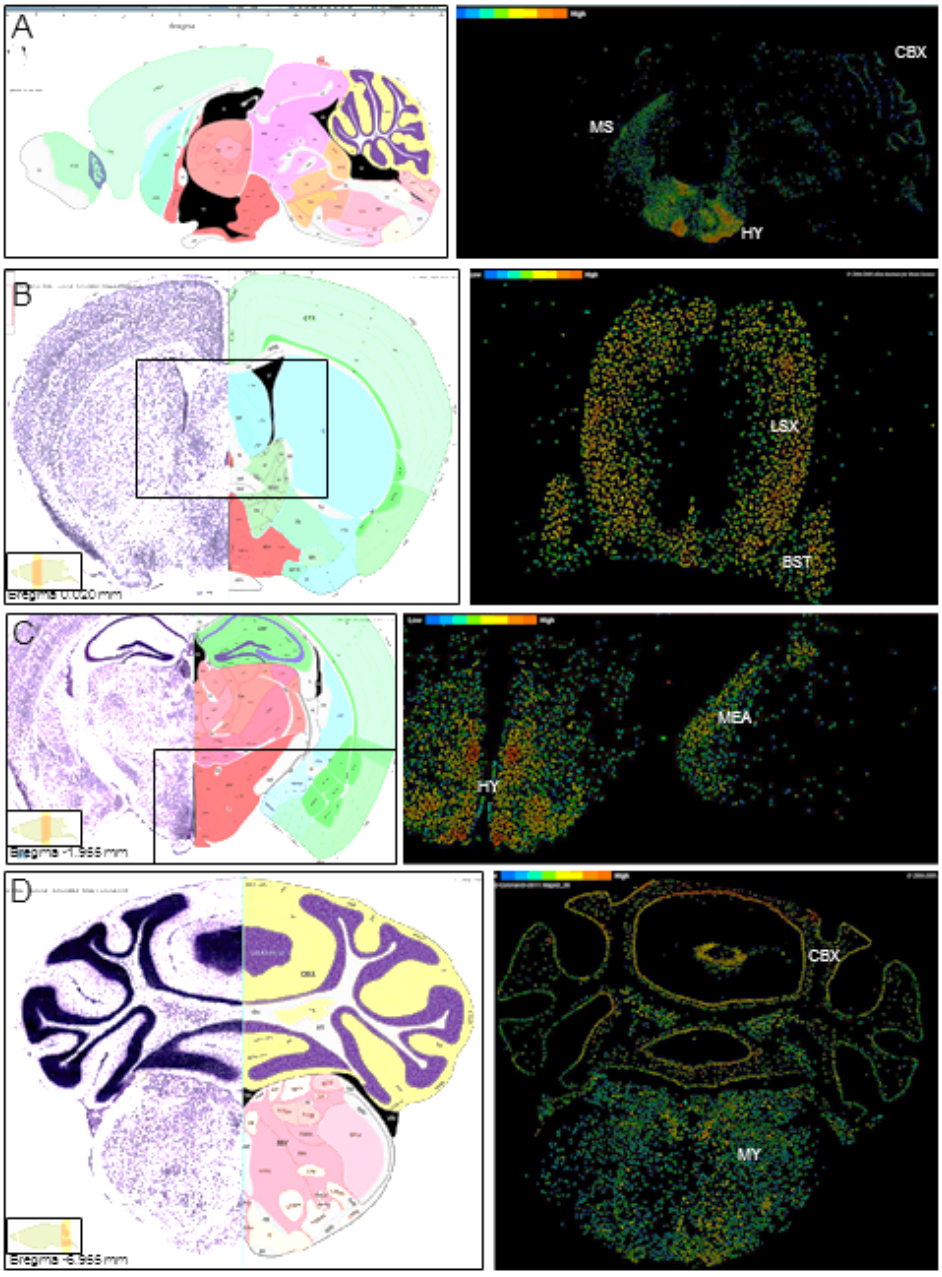

A
CBX
MS
HY
B
LSX
BST
C
MEA
HY
D
CBX
MY

Figure 2.1 Expression profile of Magel2 in the adult mouse brain by RNA in situ hybridization, retrieved from the Allen Brain Atlas.

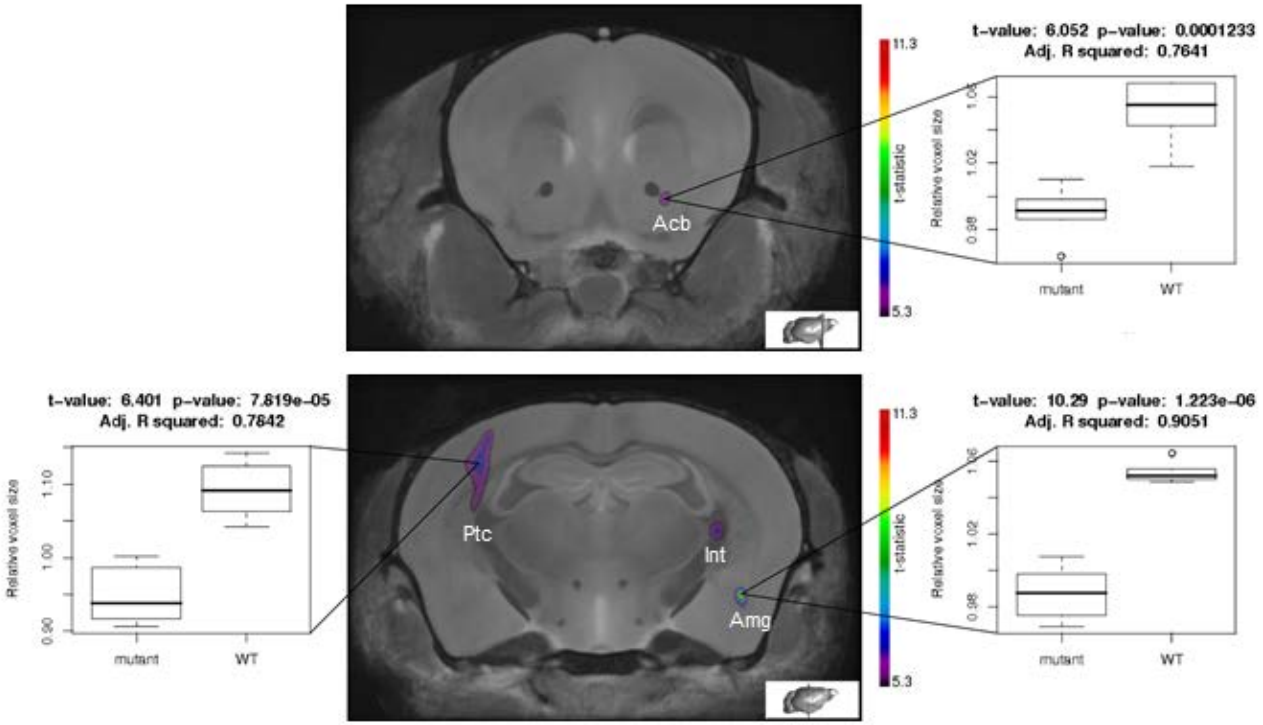
Representative sections of regions of *Magel2* expression are shown. Additional sections that display expression of *Magel2* are available from http://www.brain-map.org. The anatomical description and Nissl stained section from the Allen reference series are presented on the left, with the corresponding *Magel2*-labeled section on the right. In B-D, the coronal level of the section and distance relative to Bregma ispictorially illustrated in the bottom left corner. (A) Parasagittal section reveals intense expression in the hypothalamus (HY) including the preoptic area, the arcuate nucleus, and the paraventricular nucleus, with lesser expression in the lateral hypothalamus. The expression pattern in the medial septal nucleus and the lateral septal complex (involved in emotional responses), and bed nucleus of the stria terminalis was confirmed. The bed nucleus of the stria terminalis receives major input from the amygdala, which also expresses Magel2. Single cells in the outer region of the external granule layer of the cerebellum (CBX) also express Magel2. (B) Inset from a coronal section showing expression in the lateral septal complex (LSX) and the bed nuclei of the stria terminalis (BST) (C) Inset from a coronal section showing expression of *Magel2* in the hypothalamus (HY) and lesser expression in the medial amygdalar nucleus (MEA) (D) In the brainstem, expression was present broadly throughout the pons and the medulla (MY), and in the nucleus of the solitary tract, which relays afferent signals from cranial nerves and peripheral organs to the brainstem, hypothalamus, and cingulate gyrus. Additionally, Magel2 is expressed in the

pyramidal cell layer of the olfactory cortex piriform area, and in the hippocampal formation (CA3 pyramidal layer, dentate gyrus granular and polymorph layers, and entorhinal area of the retrohippocampus) (not shown)

2.3.3 Neuroanatomical abnormalities in Magel2-null mice measured by MRI

No gross abnormalities in sections of the *Magel2*-null mouse brains were detected by Nissl staining (not shown). To examine the brain more finely and without the confounding effect of removal from the skull, very high resolution MRI combined with computer analysis was performed. MRI analysis revealed reduced brain volume in the mutant mice (3.4% smaller than control brain, $P < 0.01$). A statistical map of the Jacobian determinant that illustrates the expansion and contraction of tissue based on genotype was used to find regions of significant change. In order to account for an inflated amount of false positive findings due to the number of statistical tests employed, the FDR technique was applied (Genovese *et al.*, 2002) with a 10% FDR threshold. This means that on average, 10% of the structures that show significant volumetric change, will be false positive (zero or one structure in our case). The following regions have a 10% or less chance of being a false positive: the parieto-temporal lobe of the cerebral cortex (representative single coronal slices in Figure 2.2), the amygdala, the dentate gyrus of the hippocampus, and the nucleus accumbens, which are regions of moderate to high *Magel2* expression as described above. The mean difference in volume for these regions was found to be significantly smaller by 4–5% in *Magel2*-null mice. Notably, no volumetric differences were present in the hypothalamus, which typically has maximal *Magel2* expression, suggesting that loss of *Magel2* expression in the hypothalamus does not cause structural changes. The corpus callosum and the olfactory bulbs were also reduced in size by 5%. No other regions of the brain showed statistically significant changes in size. The

pituitary gland was normal in size and location, as was the pineal gland, which forms part of the circadian rhythm output pathway.


t-value: 6.052 p-value: 0.0001233
Adj. R squared: 0.7641
Acb
11.3
t-statistic
5.3
Relative voxel size
1.02
0.98
mutant
WT
t-value: 6.401 p-value: 7.819e-05
Adj. R squared: 0.7842
Ptc
Int
Amg
t-value: 10.29 p-value: 1.223e-06
Adj. R squared: 0.9051
1.10
1.00
0.90

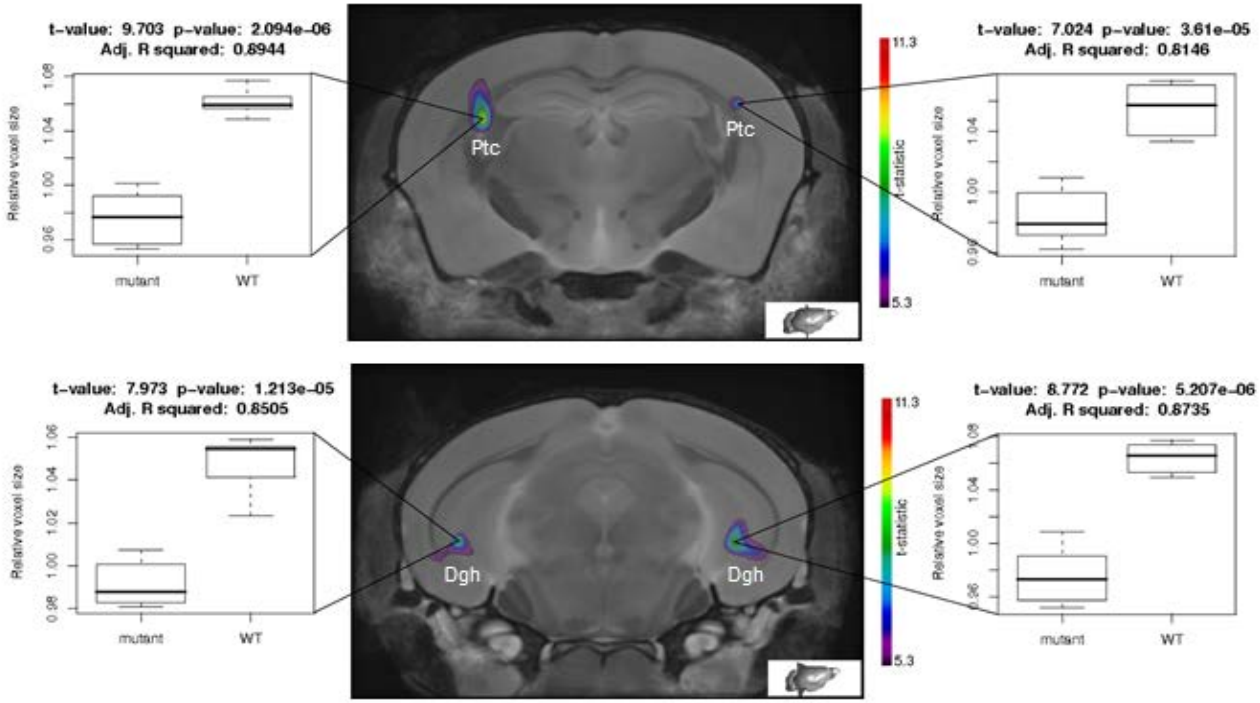

t-value: 9.703 p-value: 2.094e-06
Adj. R squared: 0.8944
Relative voxel size
mutant
WT
Ptc
Ptc
11.3
t-statistic
5.3
t-value: 7.024 p-value: 3.61e-05
Adj. R squared: 0.8146
t-value: 7.973 p-value: 1.213e-05
Adj. R squared: 0.8505
Dgh
Dgh
t-value: 8.772 p-value: 5.207e-06
Adj. R squared: 0.8735

Figure 2.2 High resolution magnetic resonance imaging of the Magel2-null adult brain.

The per-voxel results of tests of the Jacobian maps (tissue compression/expansion) are shown as composite images of four coronal sections of the *Magel2*-null (mutant) brain compared to wild-type control (WT) brain. All colored voxels are significant with a false discovery rate of 10% (n = 6 of each genotype). The coronal level of each section is pictorially illustrated in the bottom right corner. Acb, nucleus accumbens, Amg, amygdala, Dgh, dentate gyrus of the hippocampus, Int, internal capsule, Ptc, parieto-temporal lobe of the cortex.

2.3.4 Neurochemical analysis of brain regions in Magel2-null embryos and adult mice reveals altered serotonin and dopamine levels

To examine the neurochemical profile (biogenic amines and their metabolites, and amino acids) of various brain regions, high performance liquid chromatography (HPLC) with fluorimetric detection was performed (Table 2.2). Brain lysates were prepared from the cortex and cerebellum of brains from embryonic day 18 embryos and from the following brain regions of adult male *Magel2*-null and age-matched control mice: olfactory bulb, prefrontal cortex, cortex, hypothalamus, and cerebellum. No significant neurochemical differences between *Magel2*-null and control E18.5 brain samples were detected by two-way ANOVA (Table 2). A more striking neurochemical imbalance was noted in brain samples from adult male *Magel2*-null mice when analysis was performed for compounds in the catecholamine pathway (effect of genotype ($F(1,144) = 9$, $P < 0.003$)) and the indolamine pathway (effect of genotype $F(1,108) = 73$, $P < 0.0001$). On post hoc analysis, the serotonin concentration was decreased compared to control in the prefrontal cortex, cortex, and the hypothalamus, while the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) was lower in the cortex and the hypothalamus in male mice. Brain samples from adult female *Magel2*-null mice also demonstrated statistically significant reductions in serotonin concentrations to 71% (cortex) and 60% (hypothalamus) of control levels respectively. The concentration of dopamine was decreased in adult male *Magel2*-null hypothalamus, but was not significantly different from control in other regions of the brain or in the female mice. There were no neurochemical

abnormalities in *Magel2*-null cerebellum or olfactory bulb samples. Turnover rates for the catecholamine and indolamine pathways (NA/DA, DOPAC/DA, HVA/DA, 5-HT/Trypt, 5-HIAA/5-HT) were similar in the two genotypes.

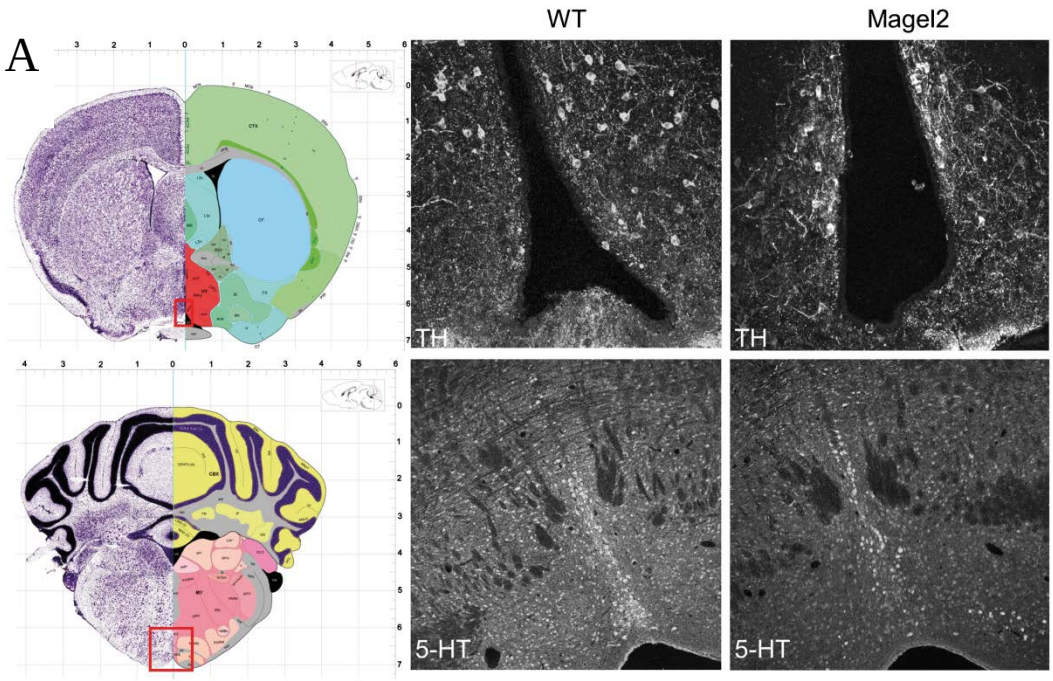
Table 2.2 Biogenic amine concentrations in brain regions from *Magel2*-null and control littermate mice

	Catecholamine pathway				Indolamine pathway		
	DA	**NA**	**DOPAC**	**HVA**	**TYRPT**	**5HT**	**5HIAA**
E18 cortex							
WT	113 ± 7	83 ± 2	16 ± 1	24 ± 1	15.8 ± 1.5	120 ± 14	256 ± 8
Magel2- null	97 ± 12	93 ± 7	22 ± 2	30 ± 2	10.8 ± 0.6	109 ± 12	286 ± 21
E18 cerebellum							
WT	52 ± 6	123 ± 10	76 ± 23	45 ± 5	16.6 ± 1.9	195 ± 19	678 ± 77
Magel2- null	54 ± 9	116 ± 15	21 ± 3	28 ± 1.2	9.2 ± 1.4	168 ± 32	335 ± 78
Adult prefrontal cortex							
WT	2658 ± 146	376 ± 24	723 ± 58	581 ± 33	3.2 ± 0.2	441 ± 21	250 ± 30
Magel2- null	2483 ± 166	359 ± 20	565 ± 93	464 ± 32	3.2 ± 0.2	369 ± 10	218 ± 20
% of WT, p-value						84%, p< 0.01	
Adult cortex							
WT	873 ± 58	375 ± 11	176 ± 11	211 ± 11	4.3 ± 0.1	377 ± 13	295 ± 16
Magel2- null	912 ± 52	317 ± 14	189 ± 21	206 ± 18	4.1 ± 0.3	282 ± 10	225 ± 9
% of WT, p-value						75%, p< 0.001	76%, p< 0.01
Adult hypothalamus							
WT	783 ± 137	587 ± 27	196 ± 42	328 ± 41	4.8 ± 0.5	528 ± 22	520 ± 19
Magel2- null	472 ± 75	557 ± 25	132 ± 24	246 ± 22	4.4 ± 0.6	372 ± 18	421 ± 13
% of WT, p-value	60%, p< 0.05					70%, p< 0.001	81%, p< 0.001

Samples are from male wild-type control (WT) and *Magel2* -null (Magel2) mice at embryonic Day 18 (E18, n =8-12 per genotype) or at 20-24 weeks of age (adult, n = 7 per genotype). Concentrations (mean ± SEM) are expressed as ng/g of tissue except for tryptophan, which is expressed as µg/g tissue. Two-way ANOVA detected a difference between genotypes in the adult samples in both the catecholamine (F (1,144) =9, $p < 0.003$) and indolamine (F (1,108) = 73, $p < 0.0001$) pathways. Bonferroni post-test p values are provided only where statistically significantly different between genotypes at $p < 0.05$.

DA, dopamine; NA, noradrenaline; DOPAC, dihydroxyphenylacetic acid; TRYPT, tryptophan; HVA, homovanillic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid.

The reduced concentration of serotonin or dopamine could be caused by a reduction in the number of dopaminergic or serotonergic neurons in the *Magel2*-null mouse brains. To examine this possibility, we first examined the location and number of dopaminergic neurons (immunoreactive with an antibody against TH and visualized by confocal microscopy) in the hypothalamus in adult and *Magel2*-null male mouse brain sections. We found a comparable number of positively staining neurons in both genotypes, and no differences in the appearance of the TH-positive neurons groups in the pre-optic region, the medial basal region, or the medial dorsal region of the hypothalamus (A11–A15 dopaminergic cell groups, Figure 2.3). Serotonergic cell groups are primarily located in the brain stem, a region of low *Magel2* expression. Examination of the location and number of neurons immunohistochemically labeled with an anti-5-HT antibody revealed no differences in the location or number of serotonergic neurons in the cell groups in the brain stem (Figure 2.3). The quantitative reduction in dopamine and serotonin content in the hypothalamus of *Magel2*-null mice therefore does not appear to be consequent to neuronal loss, consistent with our finding that there is no volumetric reduction in either the hypothalamus or the brain stem measured by structural MRI.


A
WT
Magel2
TH
TH
5-HT
5-HT

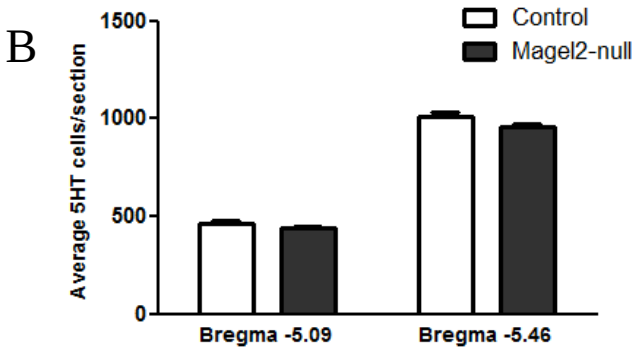

B
Control
Magel2-null
Average 5HT cells/section
1500
1000
500
0
Bregma -5.09
Bregma -5.46

Figure 2.3 Examination of dopaminergic and serotonergic neuron clusters in the Magel2-null mouse brain.

(A) Confocal microscopy of adult mouse wild-type control littermate (WT) or *Magel2*-null (Magel2) brain sections (20x magnification) labeled for dopaminergic clusters by immunohistochemistry with an antibody against tyrosine hydroxylase (top) or for serotonergic clusters with an anti-serotonin antibody (bottom). Specific brain areas examined are outlined in red on the left panels of the image. (B) Average number of serotonin expressing cells in the brainstem of control littermate and *Magel2*-null mice.

2.3.5 Magel2-null mice become less active over time in the open field, but have normal balance and strength

We determined whether behavioural abnormalities existed in adult *Magel2*-null mice that could be quantified on formal testing. Motor activity was assessed in an open field test, measuring rearing, exploratory, and fear-related behaviour (percent time in the center and the average time per crossing) in the first 5 min of the test. Off-wall rearing was increased in the *Magel2*-null mice (Figure 2.4A, $P < 0.03$), but on-wall rearing, time in the center and time per crossing were not different between genotypes (Figure 2.4A and data not shown). As expected from our previous study that measured home cage wheel running activity (Kozlov *et al.*, 2007), horizontal activity in the open field test was reduced (Figure 2.4B, $P < 0.001$), and we also observed reduced vertical activity (Figure 2.4C, $P < 0.002$). Moreover, while the control mice slightly decreased their levels of horizontal activity and increased their vertical activity over the 30 min interval, the *Magel2*-null mice became less active in both dimensions during the test. Overall, there was a significant effect of genotype on the results of the open field test ($F(1,48) = 16$, $P < 0.0002$), with the *Magel2*-null mice only about half as active as control in either the horizontal or vertical dimensions by the end of the 30 min test period.

We used the accelerating Rotarod screen to test motor coordination and balance. The *Magel2*-null mice had a 50% increase in the latency to fall off the Rotarod in a single test (Fig. 2.4D, $P < 0.02$), which was unexpected given their reduced muscle mass and overall reduced activity. The same mice were then

tested over a 3-day period to assess motor function and learning. As in the 1 day test, the *Magel2*-null mice had increased latency to fall from the Rotarod over the 3 days (Figure 2.4E, $F(1,30) = 15$, $P < 0.0006$). For both genotypes, there was increased latency on Day 3 compared to Day 1 ($P < 0.05$), and this motor learning was not different in the *Magel2*-null mice compared to control. As a further test of motor function and motivation, the time for each mouse to travel along a narrow beam to reach an enclosed safety platform was measured. There was no difference between genotypes in the traversing time, nor in the number of times the hind feet slipped off the beam. A tail suspension test is designed to test for depression-like behaviour, and is based on the observation that mice subjected to an inescapable situation will spend more time immobile when displaying depressive behaviour, but also secondarily measures strength as the mouse struggles against being suspended. The latency to first immobile period after suspension was not different between genotypes, nor was the amount of time immobilized at 6 min. Taken together, the tail suspension test, the Rotarod tests, and the beam test, and the increase in off-wall rearing in the first 5 min of the open field suggest that motor ability per se is not compromised in the *Magel2*-null mice. These tests suggest that the measurably reduced activity after habituation is not caused by deficient motor function, but may instead represent passive or anxious behaviour in the open field.

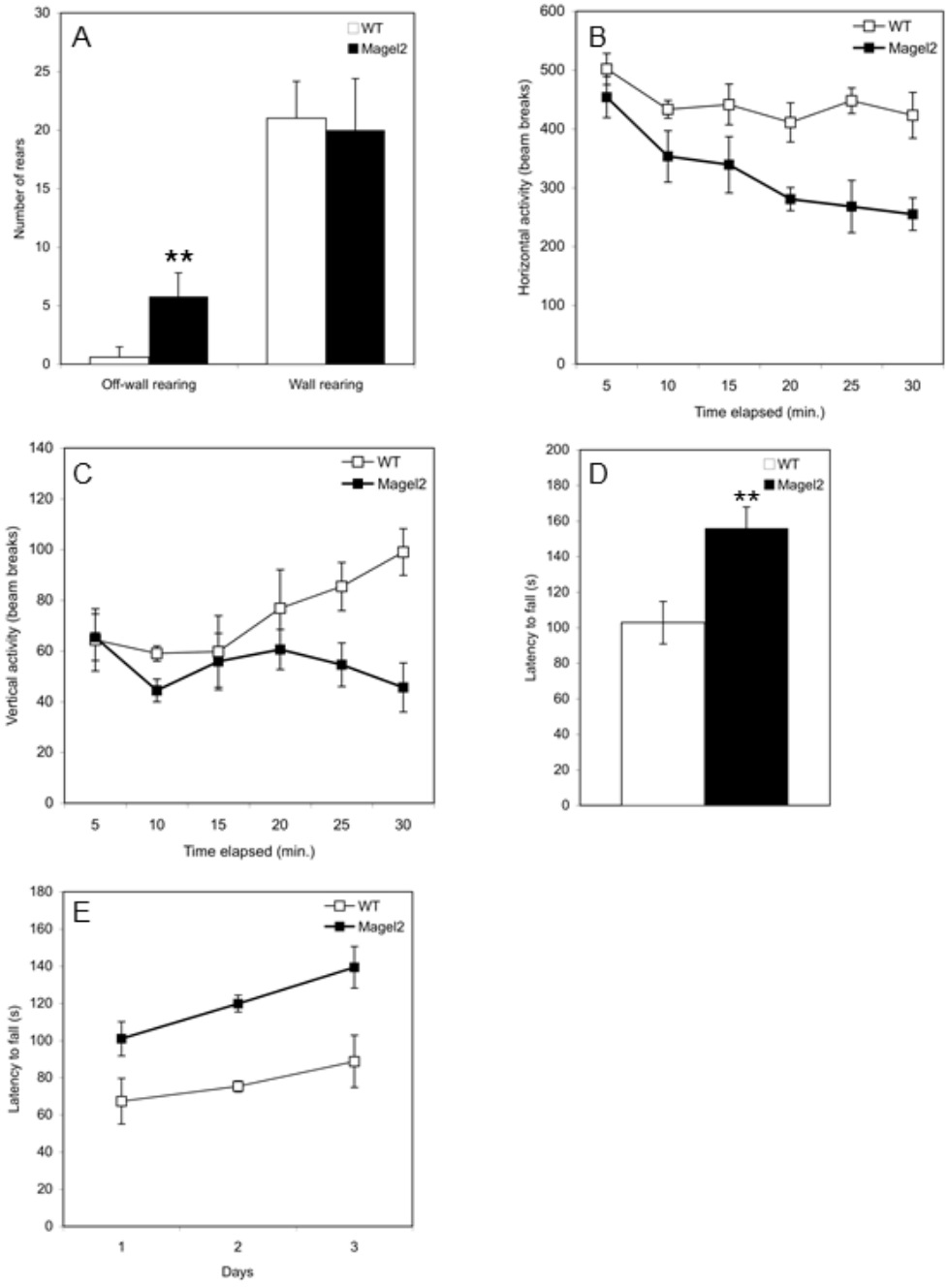

A
WT
Magel2
Number of rears
**
Off-wall rearing
Wall rearing
B
WT
Magel2
Horizontal activity (beam breaks)
Time elapsed (min.)
C
WT
Magel2
Vertical activity (beam breaks)
Time elapsed (min.)
D
WT
Magel2
**
Latency to fall (s)
E
WT
Magel2
Latency to fall (s)
Days

Figure 2.4 Motor function testing

A: Measurements of rearing either off the walls or on the walls in the first 5 min of the open field test. Data are expressed as mean ± SEM. Increased off-wall rearing in the *Magel2*-null (Magel2) mice versus wild-type control (WT) was noted; **$P < 0.05$. B,C: Horizontal and vertical activity in an open field environment over a period of 30 min, measured as number of beam breaks over 5 min intervals, are both reduced in the *Magel2*-null mice (two-way ANOVA with main effect of genotype, $P < 0.0002$). D: Latency to fall from an accelerating Rotarod during a single test is significantly increased in the *Magel2*-null mice; **$P < 0.05$. E: Latency to fall from an accelerating Rotarod increases for both genotypes over three consecutive days indicating normal motor learning. Latency to fall is significantly increased in the *Magel2*-null mice (two-way ANOVA, main effect of genotype $P < 0.0006$).

2.3.6 Tests of anxiety and learning

The elevated plus maze is a more specific test of anxiety in a novel environment. The time spent in the open or closed arms, time spent on the end of the open arms and on the central platform, time spent freezing in the open and freezing in the center, the number of passes between closed arms, the number of risk assessments, and the number of head dips were all measured, but no significant differences between genotypes were detected.

We then used a Pavlovian fear-conditioning test that measures percent of time spent with total lack of movement (freezing) after an aversive stimulus, and the learning associated with this stimulus. This test also models anticipatory anxiety, and requires a combination of amygdalar and hippocampal function. Each mouse was acclimatized in a test context, then a 30 sec auditory tone was used as the conditioned stimulus paired with an aversive unconditioned stimulus, in this case a 2 sec mild foot shock at the end of the tone. After conditioning, either the test context or the tone typically elicit a state of fear even in the absence of the foot shock, in a normal mouse. This fear is manifested as freezing, and is used to measure learning when assessed after 24 hr.

The *Magel2*-null mice tended towards an increased amount of time spent freezing during the baseline measurement on Day 1 ($1.2 \pm 0.7\%$ for control mice, $12.4 \pm 5.7\%$ for the *Magel2*-null mice, $P < 0.09$, all values expressed as mean $\pm$ standard error of the mean (SEM), Figure 2.5A). The wild-type mice exhibited an increased freezing rate after the tone (4.9-fold compared to baseline, $P < 0.05$) and after the foot shock (13.2-fold compared to baseline, $P < 0.06$), as

expected. In contrast, there was no significant change from the already high baseline level of freezing in the *Magel2*-null mice, either after the tone or after the foot shock (Figure 2.5A). The amount of freezing after the tone also remained higher in the *Magel2*-null mice compared to wild-type control (percent freezing $5.7 \pm 2.6\%$ in control mice, $23.8 \pm 7.5\%$ in *Magel2*-null mice, $P < 0.05$). After 24 hr (Day 2), each mouse was placed back into the test context used on Day 1 and freezing was measured (context in Figure 2.5A). Both genotypes increased freezing when placed in the test context on Day 2 compared to their Day 1 baseline measurement, indicating normal contextual discrimination, although the mutant mice had significantly higher absolute freezing rates ($P < 0.01$) (Figure 2.5A). Two hours later, freezing was measured in an altered context, both before (pre-tone) and after the application of a tone, to measure learning related to the cued conditioning stimulus. Similarly to the result from the baseline measurement on Day 1, the *Magel2*-null mice trended towards increased freezing in the altered context on Day 2 (pre-tone, $1.6 \pm 0.6\%$ for control mice, $6.8 \pm 2.3\%$ for the *Magel2*-null mice, $P < 0.07$). Freezing to the tone was increased compared to pre-tone freezing in both genotypes, indicating normal cued conditioning, albeit with higher absolute rates of freezing in the *Magel2*-null mice ($P < 0.001$). A two-way ANOVA analysis of all six data sets with genotype as the between factors variable revealed a significant effect of genotype on measures of freezing ($F(1,48) = 36$, $P < 0.0001$). Overall, this test indicates increased anxiety in response to novel environments or noise, but no measurable abnormality in the learning, memory, or

emotional components required for the *Magel2*-null mice to remember the association between either the context or tone and the foot shock on Day 2.

2.3.7 Magel2-null mice display altered behaviour in novel environments

As a further test to discriminate anxiety from learning, we performed a set of tests that measure reactions to novel objects in an independent cohort of male and female mice that were naïve to behavioural testing. In a one-trial object recognition task, each mouse was removed from the home cage and acclimatized to the test cage in which two objects (e.g., plastic 15 ml tube, 10 ml syringe) were placed. The total length of time needed to accumulate 38 sec of active object exploration was recorded. *Magel2*-null female mice explored the objects more actively than their control littermates, accumulating 38 sec of exploration time within 141 ± 26 sec, while the control female mice required twice as long to accumulate the total exploration time (321 ± 53 sec, $P < 0.02$) (Figure 2.5B). In contrast, the male mice showed no genotype-specific difference in exploration time. After removal of the mouse to the home cage for 5 min, one of the objects was replaced with a novel object of similar size, and the mouse was then returned to the test cage for 5 min. Wild-type mice typically spend 70% of their time with the novel object and 30% with the familiar object under this paradigm (Hammond *et al.*, 2004). These data are presented as a discrimination index (DI), the difference between the time with the novel object and the time with familiar object divided by the total exploration time. In this 5 min interval, the total time spent exploring was no longer different between genotypes for either sex, but

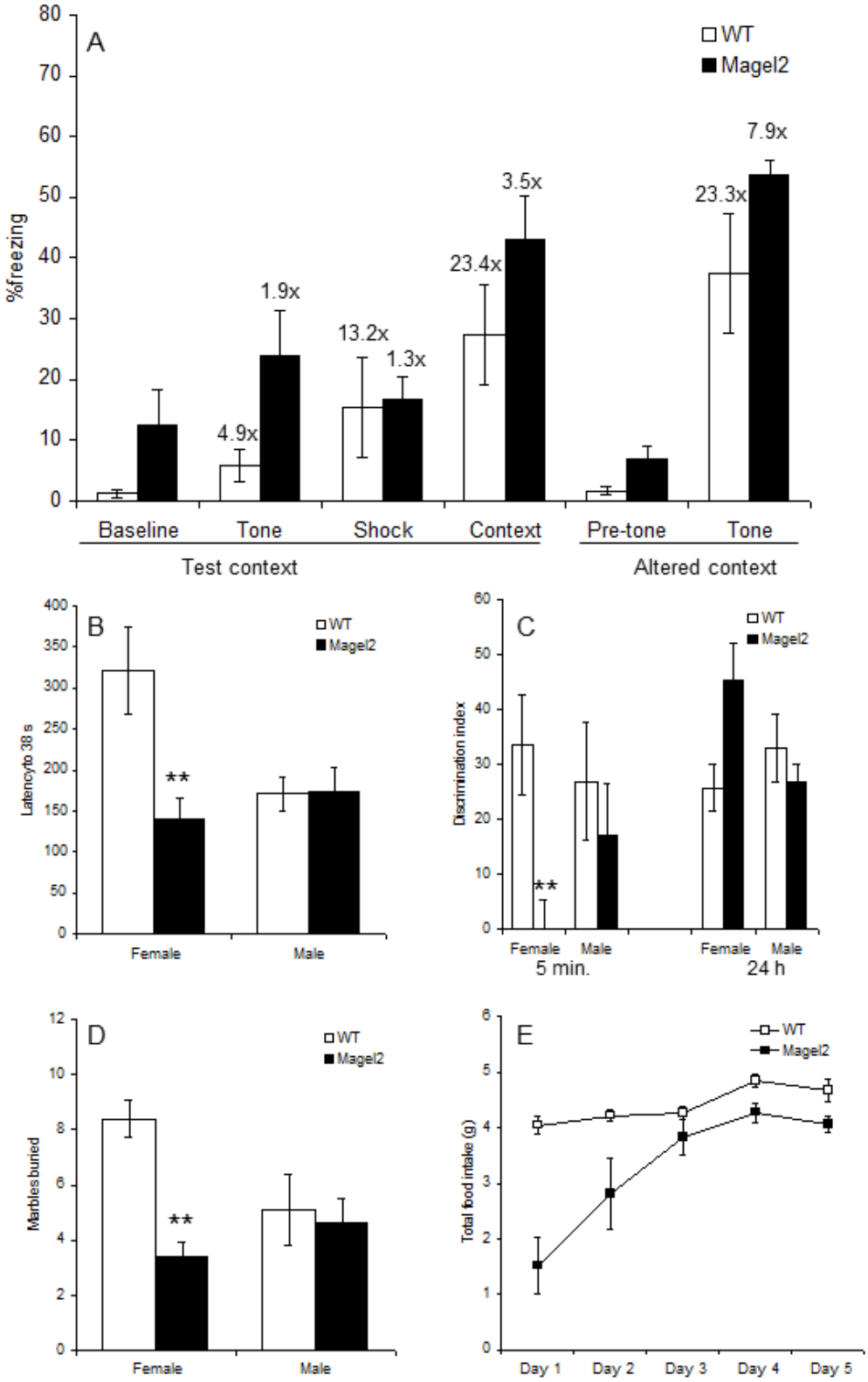

A
WT
Magel2
%freezing
80
70
60
50
40
30
20
10
0
4.9x
1.9x
13.2x
1.3x
23.4x
3.5x
23.3x
7.9x
Baseline
Tone
Shock
Context
Pre-tone
Tone
Test context
Altered context
B
WT
Magel2
Latencyto 38 s
400
350
300
250
200
150
100
50
0
**
Female
Male
C
WT
Magel2
Discrimination index
60
50
40
30
20
10
0
**
Female
Male
5 min.
Female
Male
24 h
D
WT
Magel2
Marbles buried
12
10
8
6
4
2
0
**
Female
Male
E
WT
Magel2
Total food intake (g)
6
5
4
3
2
1
0
Day 1
Day 2
Day 3
Day 4
Day 5

Figure 2.5 Fear conditioning test and novel object scenarios reveal abnormal behaviour in Magel2-null mice

A: *Magel2*-null mice freeze more than wild-type (WT) control mice in the test context at baseline and after the auditory tone, but have similar freezing rates after the foot shock. On Day 2, freezing in the familiar test context was not different between genotypes, but in contrast, *Magel2*-null mice freeze more than control at baseline in the altered context, pre-tone. Also on Day 2, the net amount of freezing in the novel context either before (pre-tone) or after (tone) the auditory tone was not different between genotypes. Data are expressed as mean ± SEM. Two-way ANOVA detected a main effect of genotype across the six freezing measurements ($P < 0.0001$). The fold change (x) in freezing during fear conditioning is also presented as a ratio of the means. Fold changes in the test context (tone, shock, Day 2 context) are compared to baseline. Fold changes in the altered context after the tone are compared to the pre-tone measurement. *Magel2*-null mice show a blunted fear reaction to either the test context or to the tone. **B**: Female *Magel2*-null display increased exploratory behaviour when placed in a cage with two novel objects, with decreased latency to reach 38 sec total exploration time. Data are expressed as mean ± SEM, $^{**}P < 0.05$. **C**: Control mice of both sexes and male *Magel2*-null mice spend more time with the novel object than with the familiar object, measured after 5 min and after 24 hr, and presented as a discrimination index (the difference between the time with the novel object and the time with familiar object divided by the total time). *Magel2*-null female mice show no preference for a novel object after 3 min (DI = 0,

$^{**}P < 0.05$). **D**: Male mice of both genotypes buried similar numbers of marbles over the three min. test interval. In contrast, female *Magel2*-null mice buried significantly fewer marbles than controls; $^{**}P < 0.05$. **E**: Control mice consume slightly less food during their first to days in an Accuscan chamber with powdered food, but increase food consumption to normal levels by Day 3 in the chamber. In contrast, *Magel2*-null mice have substantially reduced food intake when initially placed in the Accuscan chamber, and never attain normal levels of food intake.

there were differences in the rate of exploration of the novel versus the familiar object. While male and female wild-type mice and male *Magel2*-null mice preferred the novel object with this predicted preference ratio (e.g., female control mice $67 \pm 5\%$, significantly different from 50%, $P < 0.007$, $DI = 34 \pm 9$), female *Magel2*-null mice showed no preference for the novel object (preference $53 \pm 6\%$, not different from 50%, $DI = 0 \pm 5$) (Figure 2.5C). The test was repeated after 24 hr, with one of the objects identical to one from the previous day and the second a completely novel object. As in the initial test period, *Magel2*-null female mice spent more total time exploring the objects ($P < 0.03$). All classes of mice preferred the novel object (significantly different from 50%, $P < 0.01$ for all sex/genotypes, Figure 2.5C). One interpretation of this result is that object memory is intact in *Magel2*-null mice, but that *Magel2*-null female mice are averse to the new object in the 5-min test. As this task requires memory in addition to motor function and motivation (Crawley., 2008), an alternative hypothesis is that short-term memory is impaired but long-term memory is preserved in the female mutant mice.

We used a marble-burying test to further assess behaviour with novel objects, without a confounding requirement for memory. Mice will normally exhibit digging behaviour when placed in a cage with marbles on the surface of the bedding, and a number of psychotropic compounds inhibit or abolish this behaviour (Deacon., 2006). It remains controversial whether marble burying simply represents a natural behaviour in mice, or whether increased marble burying represents an anxious state (Deacon., 2006). The number of marbles

buried in a 3 min period was measured for male and female mice of both genotypes. There was no difference between genotypes in the male mice, but female *Magel2*-null mice buried significantly fewer marbles than the control mice ($P < 0.0001$, Figure 2.5D).

Our hypothesis that *Magel2*-null mice tend to avoid novel objects and are anxious in novel environments is supported by observations made during a previous feeding study. In this experiment, male mice were placed in a chamber with a powdered standard chow dispenser that measured hourly food consumption over 5 days (Bischof *et al.*, 2007). To avoid confounding the results while the mice acclimatized to the feeding system, only Days 3, 4, and 5 after placement in the new cage were used for calculation of food consumption, which was reduced to 88% of the control amount (Bischof *et al.*, 2007). In a re-analysis of the food intake data from Days 1 and 2, the *Magel2*-null mice were observed to consume only 43% of the control amount on the Day 1 (*Magel2*-null 1.7 ± 0.5 g compared with control, 4.0 ± 0.2 g, $P < 0.0005$) with three of these eight *Magel2* mice consuming less than 0.08 g of food over 24 hr (Figure 2.5E), and mutant mice consumed only 66% of wild-type levels on Day 2. These results suggest avoidance of the novel food source or anxiety in the new type of cage.

2.3.8 Additional tests of behaviour

Mice normally exhibit self-grooming behaviour, and pathological self-grooming or excessive grooming of cage mates has been interpreted as evidence of obsessive–compulsive tendencies in mice (Kalueff *et al.*, 2007). We examined grooming behaviour either under unstimulated conditions in the home cage, or by

individually spraying the mice with a fine mist of water from a hand spray bottle. Normal head to tail grooming behaviour was observed in mice of both genotypes.

2.4 Discussion

Magel2 belongs to the MAGE family of proteins, and is most closely related to MAGED1/NRAGE and necdin. Studies in cell culture and in mice have implicated necdin in neural differentiation and cell cycle exit, and structural and function deficits in the nervous system have been identified in necdin-null mice (Muscatelli *et al.*, 2000, Takazaki *et al.*, 2002, Ren *et al.*, 2003, Lee *et al.*, 2005, Tennese *et al.*, 2008). MAGED1/NRAGE inhibits cell cycle exit and facilitates p75 neurotrophin receptor-mediated apoptosis in neuronal cells (Salehi *et al.*, 2000). While necdin and MAGE-GI were shown to have activities in p75 neurotrophin receptor and E2F1-dependent pathways, similar activities could not be detected for MAGEL2, perhaps because only 490 amino acids of the 525 amino acid predicted protein (Lee *et al.*, 2000) were included in the epitope-tagged expression construct (Kuwako *et al.*, 2004). Although a cellular role for Magel2 has not been determined, abnormalities in maintenance of behavioural circadian rhythm, reduced total activity, and reduced food intake in *Magel2*-null mice have been described (Bischof *et al.*, 2007, Kozlov *et al.*, 2007). We also described decreased weight gain before weaning and increased gain after weaning leading to increased adiposity and reduced lean muscle mass in adult mice. We have now characterized the neuroanatomy, neurochemistry, and behaviour in mice null for *Magel2* and found deficits in all three areas.

2.4.1 Magel2-null mice have reduced brain volume and reduced neurotransmitter levels in discrete regions of the brain

Subtle changes in regional brain volume have been described in a variety of congenital and progressive genetic disorders, most prominently in human and mouse studies of schizophrenia (Clapcote *et al.*, 2007, van Haren *et al.*, 2008). In a recent study of mice with heritable mutations that display behavioural symptoms, 87% of these strains showed neuroimaging abnormalities including volumetric changes using high resolution MRI (Nieman *et al.*, 2007). In keeping with this finding, *Magel2*-null mice have brain abnormalities that we detected by MRI. In particular, we noted a 4% bilateral reduction in cortical volume in distinct regions of the *Magel2*-null adult mouse brain, including focused regions in the parietal-temporal lobes, and in the dentate gyrus of the hippocampus underlying the medial temporal lobe of the cortex. Notably, there were no anatomical abnormalities in the hypothalamus in *Magel2*-null mice, suggesting that the circadian rhythm deficiency in these mice is not structural in origin. These regions of volumetric reduction in the mouse are also regions of moderate to high levels of *Magel2* expression. A significant volumetric change with a magnitude of 4–5% is not atypical in mutant mice, and for example changes of a similar magnitude have been observed using high resolution MRI in a mouse model of Huntington disease (Lerch *et al.*, 2008b)and between sexes in the C57BL/6 mouse strain (Spring *et al.*, 2007). Ventriculomegaly was previously observed in Ndn-null mouse embryos (Lee *et al.*, 2005), but in contrast, brain ventricles were normally sized in *Magel2*-null mice. Notably, volumetric changes in specific brain regions

that are of comparable magnitude to that seen in the *Magel2*-null mouse brain (~5%) have been described in studies of children with autism (frontal lobe enlargement of 5–10% (Amaral *et al.*, 2008)), attention deficit hyperactivity disorder (cerebrum reductions of 3–5% (Krain and Castellanos, 2006)) and early onset schizophrenia (e.g., progressive 5–10% reduction in the prefrontal cortex (Toga *et al.*, 2006)) suggesting that this extent of volumetric change has neurologically based consequences to mammalian behaviour.

We identified neurochemical abnormalities in brain samples from adult *Magel2*-null mice, most notably reduced concentration of serotonin and its metabolite 5-HIAA to 70–80% of control levels in the prefrontal cortex, cortex and hypothalamus. We found no difference in the abundance or location of the serotonergic neuron clusters that are primarily located in the raphe nuclei of the brainstem. The rostral serotonergic group projects axons throughout the fore- and mid-brain, where reduced serotonin content was measured in the mutant mice in this study. In addition, dopamine was reduced to 60% of control values in the adult *Magel2*-null hypothalamus, although the number and location of dopaminergic neurons was not different between genotypes. Dopamine producing neurons are present in the arcuate nucleus of the hypothalamus where they project axons to the median eminence to stimulate hormone secretion from the anterior pituitary. In particular, they control the secretion of the reproductive hormone prolactin from the pituitary, and dopaminergic pathways in the hypothalamus are also critical to the regulation of growth hormone releasing hormone action on the release of growth hormone from the anterior pituitary (Garcia-Tornadu *et al.*,

2006). Dopamine concentrations were not altered in other brain regions suggesting a specific effect of the *Magel2* mutation on hypothalamic dopaminergic neurons.

It is difficult to establish a cause and effect relationship between neurochemical imbalances and behaviour in mice, particularly as total levels combine the intracellular and extracellular pools of the neurotransmitters. For example, many studies implicating serotonin in mood and behaviour use surrogate markers, such as 5-HT or 5-HIAA levels in cerebrospinal fluid or platelets, examine the activation of serotonin receptors after pharmacological intervention, or the effects of depletion of the serotonin precursor tryptophan on behaviour. Nonetheless, many studies have linked altered serotonergic and dopaminergic pathways with psychiatric disorders, most notably depression, anxiety, and self-injurious or obsessive behaviour (Hyman., 2007). Serotonin pathways have also been implicated in eating disorders (Kaye *et al.*, 2005), and serotonin is intimately involved in food intake and energy balance at least in part through modulation of the activity of hypothalamic neurons, particularly through melanocortin-dependent pathways (Heisler *et al.*, 2006). Serotonin, brain-derived neurotrophic factor, and the insulin-like growth factor cooperate in the regulation of energy metabolism and stress response in cells and whole organisms, through their roles as neurotrophic factors in the central nervous system (Mattson *et al.*, 2004). Interestingly, mice deficient for brain-derived neurotrophic factor exhibit abnormal behaviours, including obesity, anxiety, and aggression, and reduced levels of 5-HT and 5-HIAA in the adult cortex (Gaspar *et al.*, 2003). Further

studies are needed to determine whether there is a concurrent disruption of the insulin-like growth factor or brain-derived neurotrophic factor pathways in *Magel2*-null mice that could alter the balance of growth and survival of neurons.

2.4.2 Magel2-null mice are hypoactive and react abnormally to novel environments

We performed assays in *Magel2*-null mice designed to measure anxiety-like behaviour, locomotion, balance, neuromuscular function, learning, and memory. Behavioural tests in animal models can provide surrogate markers for normal or pathological human behaviour. In transgenic animal studies, interacting deficits in different processes can influence performance in behaviour tests (Crabbe and Morris, 2004, Tecott and Nestler, 2004). For example, a Rotarod test meant to assay motor function and motor learning also assesses vision, motivation not to fall, grip strength, and balance. The effects of reduced total activity, reduced muscle mass, and blunted circadian rhythm on the behaviour tests that we performed also need to be considered in our analysis.

Balance and strength were not impaired by the *Magel2* mutation, as evidenced by normal or improved function in rearing in the open field, time to cross in the beam test, increased latency to fall from the Rotarod, and equivalent time spent struggling in the tail suspension test. One interpretation of the increased latency to fall from the Rotarod is that the *Magel2*-null mice have normal strength and balance, as supported by the other tests of motor function, but have increased motivation not to fall, consistent with their abnormal reaction to other novel environments. We did however observe a significant reduction in

open field activity in *Magel2*-null mice, consistent with previous findings that used running wheels to monitor 24 hr activity of *Magel2*-null mice (Kozlov *et al.*, 2007). This reduction to about 50% of control activity, accompanied by only a 10% reduction in food consumption, causes the obesity in adult *Magel2*-null mice.

We found no evidence for learning or long-term memory deficiencies in the 3-day Rotarod test, the 24 hr novel object preference test, or the 24 hr fear-conditioning test. There was an increase in freezing in the *Magel2*-null mice on Day 2 of the fear conditioning test, suggesting they are not grossly impaired in amygdalar or hippocampal functions required for conditioned learning over 24 hr. The interpretation of learning in the fear-conditioning test was complicated by the high baseline freezing rates in the *Magel2*-null mice, which led to a smaller fold increase in freezing either to the context or to the tone on Day 2.

The conditioned fear test revealed a significant difference between genotypes not related to learning or memory: male *Magel2*-null mice have increased time spent freezing under baseline conditions, indicating increased anxiety in the test chamber. It is unlikely that reduced activity accounts for this increase in freezing during the 2 min baseline measurement, as there was only minimal difference in horizontal or vertical activity between genotypes in the first 5 min of the open field test. Rather, there was a progressive decline in activity of the *Magel2*-null mice over the following 25 min in the open field. In a test used as a proxy for anxiety in rodents, we found no inter-genotype difference in marble burying activity in male mice, nor was there any difference in the time male mice spent with objects in the cage. In contrast, female *Magel2*-null mice spent more

time exploring objects placed in a cage, did not display novel object preference when placed back into the test chamber after a 5 min interval, and were less likely to bury marbles placed on the surface of the bedding. In summary, the object-based tests with the female mice suggest a combination of poor short-term memory for novel objects and decreased motivation to manipulate objects in the cage. Interestingly, sex-specific differences in behavioural responses to novel objects were also observed in a serotonin-depletion mouse model of developmental brain disorders (Hohmann *et al.*, 2007). Further studies of anxiety-like behaviour in these mice using drugs that interact in dopaminergic and serotonergic pathways will be necessary to fully understand the extent of the behavioural deficiencies that results from loss of *Magel2*.

2.4.3 Abnormalities of brain and behaviour: comparison with PWS

In PWS, psychiatric symptoms often develop during childhood and can include mood instability, obsessive–compulsive disorder, autism spectrum disorder, cognitive rigidity, anxiety, and addictive behaviour towards to food and other substances (Cassidy and Morris, 2002, Vogels *et al.*, 2004, Soni *et al.*, 2007, Soni *et al.*, 2008). Structural changes in the brain have been detected in individuals with PWS by MRI, and include enlarged ventricles (100% of individuals tested), decreased brain volume in specific regions, particularly the parietal and occipital lobes (all children over age 5 and adults), and polymicrogyria (60%) (Miller *et al.*, 2007), while a thin corpus callosum and reduced myelination are occasionally noted (Miller *et al.*, 2007, Miller *et al.*, 1996, Miller *et al.*, 2008, Yamada *et al.*, 2006, Iughetti *et al.*, 2008). Although

abnormalities of sleep, appetite, thirst, and fertility suggest hypothalamic dysfunction, radiologically evident abnormalities of the hypothalamus have not been described to date in PWS. Imbalances of neuropeptides have been noted but not generally replicated, and brain serotonin has not been directly measured in individuals with PWS. Elevated levels of the serotonin metabolite 5-HIAA and neuropeptide Y were found in cerebrospinal fluid from a small sample of children with PWS compared to non-PWS children, although levels of 5-HIAA were comparable to matched obese children so this may not be a PWS-specific finding (Akefeldt and Mansson, 1998, Akefeldt *et al.*, 1998, Akefeldt *et al.*, 2001). Finally, a constellation of behaviour problems that includes temper tantrums, obsessive–compulsive behaviour, mood instability, skin picking, and maladaptive behaviour typifies people with PWS and sets them apart from other intellectually disabled individuals. A dysfunction of the hypothalamic-pituitary axis likely accounts for relative growth hormone deficiency, poor appetite regulation, and sleep disorders. Other aspects are thought to have mixed origins: hypogonadotropic hypogonadism may have both hypothalamic and gonadal components (Brandau *et al.*, 2008), hyperphagia stems from decreased satiation but also has components of obsessive–compulsive symptoms that may originate in the amygdala, thalamus, or orbitofrontal cortex. Maladaptive behaviour and cognitive deficiencies suggest a disorder of the frontal lobe and associated structures.

Although the extrapolation of murine studies to human behaviour must be approached with caution, studies of genetically engineered mice have successfully

recapitulated the fundamental behavioural aspects of the respective human genetic disorder in many cases (Crawley., 2008). In this case, general behavioural similarities between *Magel2*-null mice and common findings in PWS include anxiety-related traits particularly in new environments, a short-term memory deficit, and decreased voluntary activity; however, we did not identify abnormalities of learning in the mice that would parallel the intellectual disability that is essentially universal in PWS suggesting the contribution of a different gene(s) or a mouse-human difference in this respect.

Almost all individuals with PWS lack expression of multiple genes, including loss of function of MAGEL2 and NECDIN, and most PWS candidate genes are moderately to highly expressed in the brain (Lee *et al.*, 2003a). It is unclear how much contribution to the PWS phenotype is made by each of the deleted genes. Comparison with other mouse strains carrying individual PWS gene deletions may be informative, although few behavioural studies have been performed to date. The Wevrick laboratory previously showed that mice lacking necdin are underweight at birth (Pagliardini *et al.*, 2005), while another necdin-null strain has normal weight (Andrieu *et al.*, 2006). A preliminary behaviour study of the latter strain of mice showed no behavioural changes in an open field test, but clear motor and sensory deficits secondary to neuronal defects in the developing nervous system (Andrieu *et al.*, 2006). Two mouse strains lacking the snoRNAs MBII-85 suffer from post-natal mild to severe growth retardation before weaning (Skryabin *et al.*, 2007, Ding *et al.*, 2008). Behaviour of MBII-85 mutant mice is also abnormal, with differences in tests of anxiety but not working

or spatial memory, although a comparison group of similarly runted mice was not used in that study (Ding *et al.*, 2008). Thus, mice with individual deficiencies of three PWS candidate genes all have altered patterns of growth and behaviour consistent with a multigenic origin for deficits in PWS.

A report of a child with atypical PWS carrying a chromosome deletion of 175 kb (Sahoo *et al.*, 2008), and reports of individuals with PWS carrying translocations involving chromosome 15q11 that concurrently disrupt the activity of a set of small nucleolar RNAs (HBII-85) (e.g., (Schule *et al.*, 2005) and references therein), implicate the disruption of this region in the etiology of PWS while de-emphasizing the potential role of other genes. It is not possible to assess whether imprinting in the brain during pre-natal development of these rare individuals is affected by such sizeable genomic alterations within this complex imprinted region, thereby impinging on the normal expression of genes regulated by imprinting center transcript disrupted by these genomic events (Horsthemke and Buiting, 2006). Firm evidence for the relative role of specific genes in abnormalities of brain and behaviour in typical PWS therefore remains to be found. Our studies of this mouse model for *Magel2*-deficiency do however suggest an important contribution of loss of *Magel2* to circadian dysfunction and predisposition to narcolepsy, increased adiposity and reduced muscle mass, learning and behaviour, and infertility (Mercer and Wevrick, 2009), with a minor contribution to post-natal failure to thrive. Mouse models that examine the effect of ablation of individual PWS genes or these genes in combination will allow further dissection of this complex neurobehavioural condition.

2.5 Bibliography

Akefeldt,A., Ekman,R. and Mansson,J. E. (2001) "Cerebrospinal fluid neuropeptide Y in Prader-Willi syndrome." Dev.Med.Child Neurol. **43**(4):286-287.

Akefeldt,A., Ekman,R., Gillberg,C. and Mansson,J. E. (1998) "Cerebrospinal fluid monoamines in Prader-Willi syndrome." Biol.Psychiatry **44**(12):1321-1328.

Akefeldt,A. and Mansson,J. E. (1998) "Is monoamine oxidase activity elevated in Prader-Willi syndrome?" Eur.Child Adolesc.Psychiatry **7**(3):163-165.

Amaral,D. G., Schumann,C. M. and Nordahl,C. W. (2008) "Neuroanatomy of autism." Trends Neurosci. **31**(3):137-145.

Andrieu,D., Meziane,H., Marly,F., Angelats,C., Fernandez,P. A. and Muscatelli,F. (2006) "Sensory defects in Necdin deficient mice result from a loss of sensory neurons correlated within an increase of developmental programmed cell death." BMC Dev.Biol. **6**:56.

Barker,P. A. and Salehi,A. (2002) "The MAGE proteins: emerging roles in cell cycle progression, apoptosis, and neurogenetic disease." J.Neurosci.Res. **67**(6):705-712.

Bischof,J. M., Stewart,C. L. and Wevrick,R. (2007) "Inactivation of the mouse Magel2 gene results in growth abnormalities similar to Prader-Willi syndrome." Hum.Mol.Genet. **16**(22):2713-2719.

Boccaccio,I., Glatt-Deeley,H., Watrin,F., Roeckel,N., Lalande,M. and Muscatelli,F. (1999) "The human MAGEL2 gene and its mouse homologue are paternally expressed and mapped to the Prader-Willi region." Hum.Mol.Genet. **8**(13):2497-2505.

Brandau,D. T., Theodoro,M., Garg,U. and Butler,M. G. (2008) "Follicle stimulating and leutinizing hormones, estradiol and testosterone in Prader-Willi syndrome." Am.J.Med.Genet.A. **146A**(5):665-669.

Cassidy,S. B. and Morris,C. A. (2002) "Behavioral phenotypes in genetic syndromes: genetic clues to human behavior." Adv.Pediatr. **49**:59-86.

Chomez,P., De Backer,O., Bertrand,M., De Plaen,E., Boon,T. and Lucas,S. (2001) "An overview of the MAGE gene family with the identification of all human members of the family." Cancer Res. **61**(14):5544-5551.

Clapcote,S. J., Lipina,T. V., Millar,J. K., *et al.* (2007) "Behavioral phenotypes of Disc1 missense mutations in mice." Neuron **54**(3):387-402.

Clapcote,S. J., Lazar,N. L., Bechard,A. R. and Roder,J. C. (2005) "Effects of the rd1 mutation and host strain on hippocampal learning in mice." Behav.Genet. **35**(5):591-601.

Collins,D. L., Neelin,P., Peters,T. M. and Evans,A. C. (1994) "Automatic 3D intersubject registration of MR volumetric data in standardized Talairach space." J.Comput.Assist.Tomogr. **18**(2):192-205.

Crabbe,J. C. and Morris,R. G. (2004) "Festina lente: late-night thoughts on high-throughput screening of mouse behavior." Nat.Neurosci. **7**(11):1175-1179.

Crawley,J. N. (2008) "Behavioral phenotyping strategies for mutant mice." Neuron **57**(6):809-818.

Crawley,J. N. (2007)"Whats's wrong with my mouse?: Behavioral phenotyping of transgenic and knockout mice"John Wiley & Sons IncHoboken, NY.

Deacon,R. M. (2006) "Digging and marble burying in mice: simple methods for in vivo identification of biological impacts." Nat.Protoc. **1**(1):122-124.

Ding,F., Li,H. H., Zhang,S., *et al.* (2008) "SnoRNA Snord116 (Pwcr1/MBII-85) deletion causes growth deficiency and hyperphagia in mice." PLoS One **3**(3):e1709.

Dorr,A. E., Lerch,J. P., Spring,S., Kabani,N. and Henkelman,R. M. (2008) "High resolution three-dimensional brain atlas using an average magnetic resonance image of 40 adult C57Bl/6J mice." Neuroimage **42**(1):60-69.

Eiholzer,U. and Whitman,B. Y. (2004) "A comprehensive team approach to the management of patients with Prader-Willi syndrome." J.Pediatr.Endocrinol.Metab. **17**(9):1153-1175.

Garcia-Tornadu,I., Rubinstein,M., Gaylinn,B. D., *et al.* (2006) "GH in the dwarf dopaminergic D2 receptor knockout mouse: somatotrope population, GH release, and responsiveness to GH-releasing factors and somatostatin." J.Endocrinol. **190**(3):611-619.

Gaser,C., Volz,H. P., Kiebel,S., Riehemann,S. and Sauer,H. (1999) "Detecting structural changes in whole brain based on nonlinear deformations-application to schizophrenia research." Neuroimage **10**(2):107-113.

Gaspar,P., Cases,O. and Maroteaux,L. (2003) "The developmental role of serotonin: news from mouse molecular genetics." Nat.Rev.Neurosci. **4**(12):1002-1012.

Genovese,C. R., Lazar,N. A. and Nichols,T. (2002) "Thresholding of statistical maps in functional neuroimaging using the false discovery rate." Neuroimage **15**(4):870-878.

Goldstone,A. P. (2004) "Prader-Willi syndrome: advances in genetics, pathophysiology and treatment." Trends Endocrinol.Metab. **15**(1):12-20.

Gunay-Aygun,M., Schwartz,S., Heeger,S., O'Riordan,M. A. and Cassidy,S. B. (2001) "The changing purpose of Prader-Willi syndrome clinical diagnostic criteria and proposed revised criteria." Pediatrics **108**(5):E92.

Hammond,R. S., Tull,L. E. and Stackman,R. W. (2004) "On the delay-dependent involvement of the hippocampus in object recognition memory." Neurobiol.Learn.Mem. **82**(1):26-34.

Heisler,L. K., Jobst,E. E., Sutton,G. M., *et al.* (2006) "Serotonin reciprocally regulates melanocortin neurons to modulate food intake." Neuron **51**(2):239-249.

Henkelman,R., dazai,J., Lifshitz,N., *et al.* (2006) "High throughput microimaging of the mouse brain." Proc Intl Soc Magn Reson Med **14**:2010.

Hohmann,C. F., Walker,E. M., Boylan,C. B. and Blue,M. E. (2007) "Neonatal serotonin depletion alters behavioral responses to spatial change and novelty." Brain Res. **1139**:163-177.

Horsthemke,B. and Buiting,K. (2006) "Imprinting defects on human chromosome 15." Cytogenet.Genome Res. **113**(1-4):292-299.

Hyman,S. E. (2007) "Neuroscience: obsessed with grooming." Nature **448**(7156):871-872.

Iughetti,L., Bosio,L., Corrias,A., *et al.* (2008) "Pituitary height and neuroradiological alterations in patients with Prader-Labhart-Willi syndrome." Eur.J.Pediatr. **167**(6):701-702.

Kalueff,A. V., Aldridge,J. W., LaPorte,J. L., Murphy,D. L. and Tuohimaa,P. (2007) "Analyzing grooming microstructure in neurobehavioral experiments." Nat.Protoc. **2**(10):2538-2544.

Kaye,W. H., Frank,G. K., Bailer,U. F., *et al.* (2005) "Serotonin alterations in anorexia and bulimia nervosa: new insights from imaging studies." Physiol.Behav. **85**(1):73-81.

Kovacevic,N., Henderson,J. T., Chan,E., *et al.* (2005) "A three-dimensional MRI atlas of the mouse brain with estimates of the average and variability." Cereb.Cortex **15**(5):639-645.

Kozlov,S. V., Bogenpohl,J. W., Howell,M. P., *et al.* (2007) "The imprinted gene Magel2 regulates normal circadian output." Nat.Genet. **39**(10):1266-1272.

Krain,A. L. and Castellanos,F. X. (2006) "Brain development and ADHD." Clin.Psychol.Rev. **26**(4):433-444.

Kuwako,K., Taniura,H. and Yoshikawa,K. (2004) "Necdin-related MAGE proteins differentially interact with the E2F1 transcription factor and the p75 neurotrophin receptor." J.Biol.Chem. **279**(3):1703-1712.

Lee,S., Walker,C. L., Karten,B., *et al.* (2005) "Essential role for the Prader-Willi syndrome protein necdin in axonal outgrowth." Hum.Mol.Genet. **14**(5):627-637.

Lee,S., Walker,C. L. and Wevrick,R. (2003a) "Prader-Willi syndrome transcripts are expressed in phenotypically significant regions of the developing mouse brain." Gene Expr.Patterns **3**(5):599-609.

Lee,S., Walker,C. L. and Wevrick,R. (2003b) "Prader-Willi syndrome transcripts are expressed in phenotypically significant regions of the developing mouse brain." Gene Expr.Patterns **3**(5):599-609.

Lee,S., Kozlov,S., Hernandez,L., *et al.* (2000) "Expression and imprinting of MAGEL2 suggest a role in Prader-willi syndrome and the homologous murine imprinting phenotype." Hum.Mol.Genet. **9**(12):1813-1819.

Lein,E. S., Hawrylycz,M. J., Ao,N., *et al.* (2007) "Genome-wide atlas of gene expression in the adult mouse brain." Nature **445**(7124):168-176.

Lerch,J. P., Carroll,J. B., Dorr,A., *et al.* (2008a) "Cortical thickness measured from MRI in the YAC128 mouse model of Huntington's disease." Neuroimage **41**(2):243-251.

Lerch,J. P., Carroll,J. B., Spring,S., *et al.* (2008b) "Automated deformation analysis in the YAC128 Huntington disease mouse model." Neuroimage **39**(1):32-39.

Mattson,M. P., Maudsley,S. and Martin,B. (2004) "A neural signaling triumvirate that influences ageing and age-related disease: insulin/IGF-1, BDNF and serotonin." Ageing Res.Rev. **3**(4):445-464.

Mercer,R. E. and Wevrick,R. (2009) "Loss of magel2, a candidate gene for features of prader-willi syndrome, impairs reproductive function in mice." PLoS ONE **4**(1):e4291.

Miller,J. L., Goldstone,A. P., Couch,J. A., *et al.* (2008) "Pituitary abnormalities in Prader-Willi syndrome and early onset morbid obesity." Am.J.Med.Genet.A. **146A**(5):570-577.

Miller,J. L., Couch,J. A., Schmalfuss,I., He,G., Liu,Y. and Driscoll,D. J. (2007) "Intracranial abnormalities detected by three-dimensional magnetic resonance imaging in Prader-Willi syndrome." Am.J.Med.Genet.A. **143**(5):476-483.

Miller,L., Angulo,M., Price,D. and Taneja,S. (1996) "MR of the pituitary in patients with Prader-Willi syndrome: size determination and imaging findings." Pediatr.Radiol. **26**(1):43-47.

Moore,R. Y., Speh,J. C. and Leak,R. K. (2002) "Suprachiasmatic nucleus organization." Cell Tissue Res. **309**(1):89-98.

Muscatelli,F., Abrous,D. N., Massacrier,A., *et al.* (2000) "Disruption of the mouse Necdin gene results in hypothalamic and behavioral alterations reminiscent of the human Prader-Willi syndrome." Hum.Mol.Genet. **9**(20):3101-3110.

Nieman,B. J., Lerch,J. P., Bock,N. A., Chen,X. J., Sled,J. G. and Henkelman,R. M. (2007) "Mouse behavioral mutants have neuroimaging abnormalities." Hum.Brain Mapp. **28**(6):567-575.

Nieman,B. J., Flenniken,A. M., Adamson,S. L., Henkelman,R. M. and Sled,J. G. (2006) "Anatomical phenotyping in the brain and skull of a mutant mouse by magnetic resonance imaging and computed tomography." Physiol.Genomics **24**(2):154-162.

Pagliardini,S., Ren,J., Wevrick,R. and Greer,J. J. (2005) "Developmental abnormalities of neuronal structure and function in prenatal mice lacking the prader-willi syndrome gene necdin." Am.J.Pathol. **167**(1):175-191.

Parent,M., Bush,D., Rauw,G., Master,S., Vaccarino,F. and Baker,G. (2001) "Analysis of amino acids and catecholamines, 5-hydroxytryptamine and their metabolites in brain areas in the rat using in vivo microdialysis." Methods **23**(1):11-20.

Ren,J., Lee,S., Pagliardini,S., *et al.* (2003) "Absence of Ndn, encoding the Prader-Willi syndrome-deleted gene necdin, results in congenital deficiency of central respiratory drive in neonatal mice." J.Neurosci. **23**(5):1569-1573.

Rogers,D. C., Fisher,E. M., Brown,S. D., Peters,J., Hunter,A. J. and Martin,J. E. (1997) "Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment." Mamm.Genome **8**(10):711-713.

Sahoo,T., del Gaudio,D., German,J. R., *et al.* (2008) "Prader-Willi phenotype caused by paternal deficiency for the HBII-85 C/D box small nucleolar RNA cluster." Nat.Genet. **40**(6):719-721.

Salehi,A. H., Roux,P. P., Kubu,C. J., *et al.* (2000) "NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and facilitates nerve growth factor-dependent apoptosis." Neuron **27**(2):279-288.

Schule,B., Albalwi,M., Northrop,E., *et al.* (2005) "Molecular breakpoint cloning and gene expression studies of a novel translocation t(4;15)(q27;q11.2) associated with Prader-Willi syndrome." BMC Med.Genet. **6**:18.

Skryabin,B. V., Gubar,L. V., Seeger,B., *et al.* (2007) "Deletion of the MBII-85 snoRNA gene cluster in mice results in postnatal growth retardation." PLoS Genet. **3**(12):e235.

Soleimani,L., Roder,J. C., Dennis,J. W. and Lipina,T. (2008) "Beta N-acetylglucosaminyltransferase V (Mgat5) deficiency reduces the depression-like phenotype in mice." Genes Brain Behav. **7**(3):334-343.

Soni,S., Whittington,J., Holland,A. J., *et al.* (2008) "The phenomenology and diagnosis of psychiatric illness in people with Prader-Willi syndrome." Psychol.Med. **38**(10):1505-1514.

Soni,S., Whittington,J., Holland,A. J., *et al.* (2007) "The course and outcome of psychiatric illness in people with Prader-Willi syndrome: implications for management and treatment." J.Intellect.Disabil.Res. **51**(Pt 1):32-42.

Spring,S., Lerch,J. P. and Henkelman,R. M. (2007) "Sexual dimorphism revealed in the structure of the mouse brain using three-dimensional magnetic resonance imaging." Neuroimage **35**(4):1424-1433.

Takazaki,R., Nishimura,I. and Yoshikawa,K. (2002) "Necdin is required for terminal differentiation and survival of primary dorsal root ganglion neurons." Exp.Cell Res. **277**(2):220-232.

Tecott,L. H. and Nestler,E. J. (2004) "Neurobehavioral assessment in the information age." Nat.Neurosci. **7**(5):462-466.

Tennese,A. A., Gee,C. B. and Wevrick,R. (2008) "Loss of the Prader-Willi syndrome protein necdin causes defective migration, axonal outgrowth, and survival of embryonic sympathetic neurons." Dev.Dyn. **237**(7):1935-1943.

Toga,A. W., Thompson,P. M. and Sowell,E. R. (2006) "Mapping brain maturation." Trends Neurosci. **29**(3):148-159.

Tyszka,J. M., Readhead,C., Bearer,E. L., Pautler,R. G. and Jacobs,R. E. (2006) "Statistical diffusion tensor histology reveals regional dysmyelination effects in the shiverer mouse mutant." Neuroimage **29**(4):1058-1065.

van Haren,N. E., Bakker,S. C. and Kahn,R. S. (2008) "Genes and structural brain imaging in schizophrenia." Curr.Opin.Psychiatry. **21**(2):161-167.

Vogels,A., De Hert,M., Descheemaeker,M. J., *et al.* (2004) "Psychotic disorders in Prader-Willi syndrome." Am.J.Med.Genet.A. **127A**(3):238-243.

Yamada,K., Matsuzawa,H., Uchiyama,M., Kwee,I. L. and Nakada,T. (2006) "Brain developmental abnormalities in Prader-Willi syndrome detected by diffusion tensor imaging." Pediatrics **118**(2):e442-8.

Chapter 3: Loss of *Magel2*, a Candidate Gene for Features of Prader-Willi Syndrome, Impairs Reproductive Function in Mice

This chapter is a modified version of a previously published manuscript

Mercer, R.E., and Wevrick, R. (2009) Loss of *Magel2*, a Candidate Gene for Features of Prader-Willi Syndrome, Impairs Reproductive Function in Mice. *PLoS One.* 4(1):e4291.

With the exception of testosterone, LH, and FSH assays, R.E. Mercer completed all the experiments in this chapter. These assays were performed by the University of Virginia Centre for Research in Reproduction Ligand Assay and Analysis Core.

3.1 Introduction

The master pacemaker of circadian rhythm lies in the suprachiasmatic nucleus (SCN) of the hypothalamus. Neurons in the SCN send axonal projections to hypothalamic and non-hypothalamic target regions involved in reproduction, including the medial preoptic area, the gonadotropin-releasing hormone (GnRH) neurons, and the autonomic nervous system. Alterations to circadian rhythm, either through surgical hypothalamic lesions or genetic mutation, have downstream effects on circadian behaviour and hormonal function. In animal models, circadian disruption is often associated with a decrease in reproductive performance. Mutations in key circadian rhythm genes affect female fertility through irregular estrous cycles, pregnancy failure, and reduced survival to weaning in rodents (Miller *et al.*, 2004, Kennaway *et al.*, 2004, Boden and Kennaway, 2006). Male circadian mutant mice typically have normal reproductive capacity, although *Bmal1* deficiency can cause infertility (Alvarez *et al.*, 2008) and *clockΔ19* male mice sire smaller litters (Dolatshad *et al.*, 2006). A disruption of the circadian output gene VPAC2R causes an age-related decline in male fertility associated with seminiferous tubular degeneration and associated hypospermia (Asnicar *et al.*, 2002).

Altered patterns of sleep and decreased reproductive capacity coincide in several disabling human genetic disorders, including Prader-Willi syndrome (PWS), Smith-Magenis syndrome, and Fragile-X syndrome. PWS is a contiguous gene deletion syndrome generally recognized at birth because of severe hypotonia, hypogonadism, and failure to thrive, followed by developmental delay,

hyperphagic obesity, and relative growth hormone deficiency in early childhood (Gunay-Aygun *et al.*, 2001). Hypogonadotropic hypogonadism typically manifests as hypoplastic external genitalia and delayed gonadal maturation, with delayed menarche and oligomenorrhea in females, and cryptorchidism and hypogonadism in males (Eiholzer *et al.*, 2006). While there are two reports of successful pregnancies in women with PWS following hormonal induction, male fertility has never been reported (Eiholzer *et al.*, 2006, Burman *et al.*, 2001, Crino *et al.*, 2003). Excessive daytime sleepiness and nighttime sleep disruptions frequently occur in PWS, although disruption of the circadian rhythm *per se* has not been described.

People with PWS have congenital loss of function of at least five genes including *MAGEL2*, encoding a member of the MAGE family of proteins (Lee *et al.*, 2000, Boccaccio *et al.*, 1999). In mice, *Magel2* is highly expressed in the hypothalamus, and *Magel2* RNA has a circadian profile of expression in the SCN (Kozlov *et al.*, 2007, Lee *et al.*, 2003). Mice with a targeted deletion of *Magel2* have altered circadian patterns of food consumption and wheel running activity that point to a deficiency in circadian output from the SCN (Kozlov *et al.*, 2007). We noted reduced weight gain between birth and weaning and increased adiposity in adult *Magel2*-null mice, with reduced food intake and activity levels (Bischof *et al.*, 2007). We postulated that the hypothalamic defect that we propose causes abnormalities of circadian rhythm and metabolism in *Magel2*-null mice could be accompanied by reduced fertility. We now report that loss of *Magel2* alters reproductive function in both male and female mice.

3.2 Materials and Methods

Mouse breeding and handling

Animal procedures were approved by the University of Alberta Animal Policy and Welfare Committee. The *Magel2* mouse colony was maintained on a C57Bl/6 background by breeding Magel2−/+ female mice carrying a maternally inherited *Magel2-lacZ* knock-in allele with C57Bl/6 male mice to generate heterozygous, functionally wild-type offspring. Because of imprinting that silences the maternally inherited allele, *Magel2*-null mice retain expression only from the paternally inherited *lacZ* knock-in allele (Kozlov *et al.*, 2007). C57Bl/6 female mice were bred with Magel2−/+ male mice carrying a maternally inherited *Magel2-lacZ* knock-in allele. This cross generated *Magel2* +/− mice carrying a paternally inherited *lacZ* knock-in allele (*Magel2*-null, no expression of *Magel2*) and *Magel2* +/+ (control littermate) offspring. Mice were genotyped from ear notch biopsies as described in Chapter 2. Mice were weaned between three and four weeks of age unless otherwise stated and then housed 3–4 per cage with food (PicoLab Mouse Diet 20, LabDiet) and water *ad libitum*, and maintained under 12:12 light dark conditions.

Puberty and estrous cycle examinations

Female mice used for timing of puberty were weaned at 21 days of age, and inspected daily for the occurrence of vaginal opening. Beginning on the day of vaginal opening, vaginal smears were obtained from the mice using a fine tipped swab as described (Goldman *et al.*, 2007). Smears were briefly stained in Modified Wright Stain (WS16, Sigma-Aldrich, St. Louis, MO, USA) and

examined under a light microscope, with the observer blind to the genotype of the mouse. Smears were characterized as diestrus if mostly polymorphonuclear leukocytes were present, proestrus when mostly small nucleated and some cornified cells were present, and estrus when the smear was completely cornified. Age at first estrus was recorded as the day when the first fully cornified smear occurred. Beginning at 9 or 26 weeks of age, daily vaginal smears were taken to monitor estrous cycles, for a minimum of 21 days. Representative data from individual mice are shown in Figure 3.4. For fertility measurements, mice were paired at specific ages and monitored for pregnancy. Males were removed when the female was visibly pregnant, and the cage monitored for the timing of birth of the litter.

Gonadal histology

Testes and ovaries were dissected from *Magel2*-null and control mice at 10 and 24–26 weeks of age. Tissues were fixed in 10% neutral buffered formalin (Sigma-Aldrich HT50-1-1, St. Louis, MO, USA), processed into paraffin, sectioned at 5–8 µm, and stained with hematoxylin and eosin for histological analysis. Sections were prepared by the Histology Core Facility of the Alberta Diabetes Institute, Edmonton, Alberta.

Immunohistochemistry of GnRH in the brain

For immunohistochemistry, brains from 10- or 26-week old control and *Magel2*-null mice were perfused and fixed in 4% paraformaldehyde, and cut coronally into 150 µm sections using a vibratome. Sections were left at 4°C in 25% sucrose in PBS for 24 h to dehydrate. Sections were washed 3 times with

PBS, and then incubated with a GnRH antibody (PA1-121, Affinity Bioreagents, Golden, CO, USA, diluted 1:1000) in 2% normal goat serum with 0.3% TritonX-100 at 4°C with gentle shaking for 48 h. Sections were washed in PBS, and then incubated with a goat anti-rabbit secondary antibody labeled with Alexa Fluor 594 (Invitrogen, Carlsbad, CA, USA) at 1:1000 dilution in 2% normal goat serum and 0.3% TritonX-100 for 2 h at room temperature in the dark. Sections were then washed thoroughly with PBS, mounted on slides using ProLong Gold (Invitrogen, Carlsbad, CA, USA), and analyzed by confocal microscopy.

Hormonal assays

Blood for analysis of reproductive hormones was obtained by cardiac puncture. Serum was purified by centrifugation, and stored at −20°C until radioimmunoassay was performed. Testosterone, LH, and FSH levels were analyzed by the University of Virginia Centre for Research in Reproduction Ligand Assay and Analysis Core (supported by the Eunice Kennedy Shriver NICHD/NIH Grant U54-HD28934).

Olfaction tests

On the first day of the buried food test, a $2cm^3$ piece of sharp cheddar cheese was placed in each mouse's home cage. The following day, mice were subjected to a 24 h fast beginning just before lights out. After this 24 h period, the mice were habituated to clean 29×19×13 cm filter top cages with approximately 5 cm deep bedding for a minimum of 20 min. The mouse was then briefly removed and an approximately $2cm^3$ piece of sharp cheddar cheese was buried under the bedding with random placement along a cage side, avoiding the corners. The

mouse was reintroduced to the cage and latency to find the cheese was recorded. Each mouse was tested three times with a minimum 48 h period separating test periods.

A three choice olfactory preference test was administered to assess pheromone odor preference. Each mouse was tested in a clean 48×26×21 cm plexiglass cage containing 3 small (7×7×3 cm) containers filled with either clean bedding (unused autoclaved wood shavings), male-soiled bedding (collected and pooled from cages containing singly housed sexually experienced C57BL/6 males), or female-soiled bedding (collected and pooled from cages housing 3–4 female mice with at least 2 in the active state of estrous as assessed by vaginal appearance (Champlin *et al.*, 1973)). Bedding was used on the same day it was collected. These containers were placed randomly on either side and in the centre of the cage. Mice were habituated to the test arena for a minimum of 10 min. prior to introduction of the olfactory stimuli. The time spent by each mouse investigating each type of bedding was recorded over a 10 min. time period. For each mouse, the time spent with each of the three containers was divided by the total time spent investigating the containers to arrive at a percent time with each choice of bedding.

Statistical analysis

Fertility rates were analyzed by Fisher's exact test. Testosterone, LH, and FSH levels were compared using a two-tailed Mann-Whitney U test. Otherwise, statistical analyses of differences between genotypes were performed using the two-way ANOVA function of the GraphPad Prism 4 software package, or

Student's paired t-tests. Differences with $p<0.05$ after correction for multiple t-testing were considered significant.

3.3 Results

Magel2-null mice were previously constructed by gene-targeted replacement of the open reading frame of *Magel2* with a lacZ reporter cassette (Kozlov *et al.*, 2007), and are maintained on a C57BL/6 background. Mice that inherit the gene-targeted allele from their fathers are "*Magel2*-null" and lack expression of *Magel2* because of genomic imprinting that silences the maternally inherited wild-type allele. Young *Magel2*-null mice are relatively healthy and fertile and display no overt physiological abnormalities, but do display abnormal behaviour on formal testing (Mercer *et al.*, 2009). We noted no differences in the anatomy of the external or internal reproductive organs of *Magel2*-null mice at birth, and in particular did not detect any male mice with cryptorchidism.

3.3.1 Magel2-null females display delayed and lengthened puberty

Female mice were monitored for puberty by inspection for vaginal opening and age at first estrus. *Magel2*-null females display a slight but significant delay of 1.4 days in age at vaginal opening (Table 3.1, $p<0.0004$) and an additional delay of 5.3 days in age at first estrus (Table 3.1, $p<0.008$) indicating defects in both the initiation and duration of puberty. It has been established that rodents that are underweight at the normal time of puberty can exhibit delayed vaginal opening and delayed onset of estrus (Kennedy and Mitra, 1963). Although *Magel2*-null pups are underweight prior to weaning (Bischof *et*

al., 2007) no significant differences in the weights of the pups were observed during the time of pubertal examination (P28–P40).

Table 3.1 Onset of puberty based on vaginal opening and first estrus

	Control (n)	***Magel2*-null (n)**
Age at vaginal opening (d)	29.5 ± 0.31 (18)	30.9 ± 0.18 *(17)
Age at first estrus (d)	31.3 ± 0.87 (7)	36.6 ± 1.41** (7)

Data shown are mean ± S.E.M., differences between control and *Magel2*-null were significant(*$p<0.0004$, **$p<0.008$)

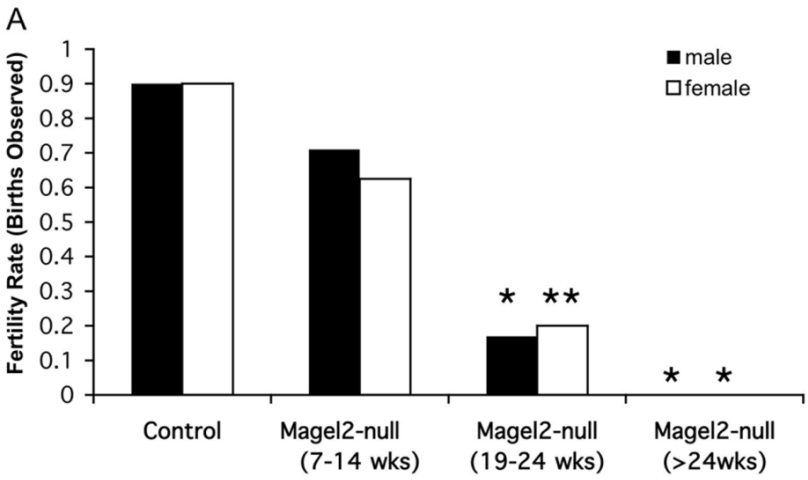
3.3.2 Magel2-null mice show early reproductive decline with infertility by 24 weeks of age

To test whether loss of *Magel2* affects fertility, we paired either *Magel2*-null mice or their control littermates with 6–10 week old C57BL/6 mice. We then noted whether a litter was born, and the number of days until the litter was born, with pairs split when the female was visibly pregnant. The fertility rate for pairings between control littermate mice and C57BL/6 mice was over 80% (Figure 3.1A, control littermates aged 7–35 weeks grouped together). In contrast, pairings between *Magel2*-null and C57BL/6 mice were less successful (Figure 1A, *Magel2*-null mice split into three age categories). While 71% of C57BL/6 females paired to 7 to 14-week old *Magel2*-null males became pregnant, significantly fewer (17%, $p<0.001$) of C57BL/6 females paired with 19 to 24-week old *Magel2*-null male mice became pregnant. Furthermore, no pregnancies

have been observed in C57BL/6 females paired with *Magel2*-null males older than 24 weeks of age ($p<0.001$, Figure 3.1A, n = 12 in each age category). *Magel2*-null females were also less fertile and displayed declining fertility with age. Although 63% of 7 to 14-week old *Magel2*-null females became pregnant when paired with C57BL/6 males, significantly fewer (20%, $p<0.01$) 19 to 24-week old *Magel2*-null females became pregnant, and no litters have been born to mutant females paired beyond 24 weeks of age, even when housed with fertile C57BL/6 males for over 60 days ($p<0.001$, Figure 3.1A, n = 8–10 in each category).

Furthermore, for both male and female *Magel2*-null mice that were eventually successful in breeding, the interval between pairing and birth was significantly extended at all ages. Daily examination of the female mice for the presence of vaginal plugs indicative of mating revealed that as with C57BL/6 mice, most *Magel2*-null females mated with C57BL/6 males and C57BL/6 females mated with *Magel2*-null males had litters within 20 days of the positive plug date, indicating that there was no decreased survival of entire litters. Rather, the mean number of days between pairing and mating was extended to 9 days for pairings involving *Magel2*-null males, compared to the C57BL/6 mean of 4 days that is consistent with the estrous cycle of wild-type female mice (Figure 3.1B, n = 14 of each genotype). For matings involving *Magel2*-null females, the interval between pairing and mating was further extended to a mean of 12 days (Figure 3.1B, n = 12 of each genotype).

The mean litter size sired by *Magel2*-null males was similar to the size of litters sired by functionally wild-type *Magel2*$^{-/+}$ male mice carrying a maternally inherited *Magel2-lacZ* knock-in allele, suggesting adequate number of sperm and comparable embryonic viability. Ninety-six percent of pups sired by *Magel2*-null males survive until weaning, a number consistent with the typical 95% weaning rate of control mice in the same environment. In contrast, the average litter size born to *Magel2*-null females was slightly but significantly smaller than controls (*Magel2*-null 6.4 pups versus control 7.8 pups, $p<0.05$, n = 10–15 litters per genotype), indicating fewer ovulations or increased embryo resorptions. The *Magel2*-null females frequently cannibalized their litters within two days of birth, and we had no litters that survived to weaning born to *Magel2*-null females older than 10 weeks of age (n = 8). Of female *Magel2*-null mice younger than 10 weeks old, we were only able to successfully wean two litters to *Magel2*-null females in our conventional breeding facility, and survival to weaning was 50–60%, much less than the typical weaning rate of 95%. Surviving pups from *Magel2*-null dams were of normal size and weight at weaning, suggesting intact ability of *Magel2*-null female mice to lactate and foster offspring despite low rates of survival of the pups.


A
Fertility Rate (Births Observed)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
male
female
* **
* *
Control
Magel2-null (7-14 wks)
Magel2-null (19-24 wks)
Magel2-null (>24wks)

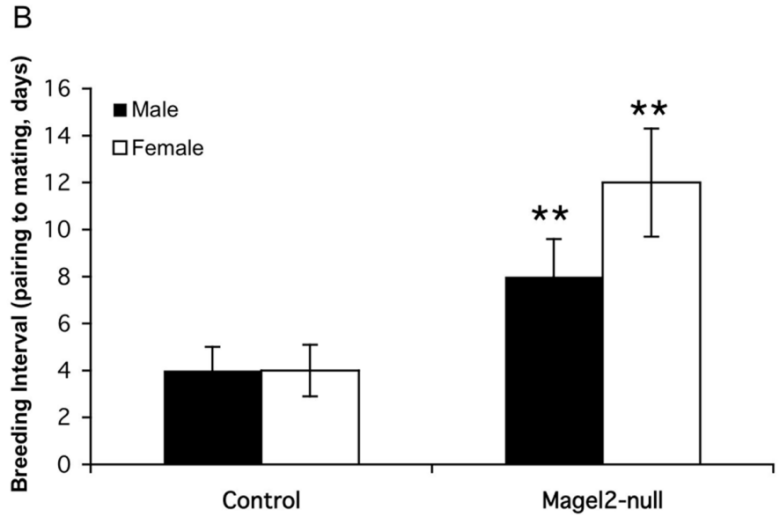

B
Breeding Interval (pairing to mating, days)
16
14
12
10
8
6
4
2
0
Male
Female
**
**
Control
Magel2-null

Figure 3.1 Reduced fertility in *Magel2*-null mice compared to controls.

(A) Fertility rate was measured by pairing mice and monitoring the cages for births, with pairs split when the female mouse was visibly pregnant. The fertility rate is the percentage of pairings that resulted in a litter, for each age group. The control littermate fertility rate includes mice aged 7–35 weeks. The fertility rate for *Magel2*-null mice is split into three age categories as marked. All mice tested were paired with C57BL/6 mice that were 6–10 weeks old. Declining fertility in the *Magel2*-null mice of both sexes is evident by 19 weeks of age, with infertility beyond 24 weeks of age. (B) Breeding interval was determined by subtracting gestational length from the number of days between pairing and birth. Extended breeding intervals are seen in both male and female *Magel2*-null mice. *$p<0.001$; **$p<0.01$ for Magel2-null versus control, error bars represent SEM.

3.3.3 Magel2-null males have reduced testosterone, but normal leutinizing hormone and follicle-stimulating hormone levels

Magel2 is expressed predominantly in the brain, so effects on reproductive systems presumably originate in the nervous system. Hypothalamic regulation of reproductive function is controlled by GnRH neurons, which stimulate the release of leutinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. To rule out an intrinsic GnRH neuron deficit, we sectioned perfused brains of 10 week-old female mice, and both 10 and 24 week-old male mice, then performed immunohistochemistry with a GnRH antibody. No difference was observed in neuron number or GnRH content between the *Magel2*-null and control brains at either age, indicating that loss of *Magel2* does not cause loss or displacement of GnRH neurons.

We next measured key reproductive hormones controlled by GnRH secretion in a group of 20–26 week-old male mice. Mean serum testosterone levels were significantly lower *Magel2*-null mice (*Magel2*-null 6.1±1.4 ng/ml versus control 20.2±9.9 ng/ml, $p<0.03$). Subsequent measurement of LH and FSH levels revealed low-normal LH levels (*Magel2*-null 0.12±0.03 ng/ml versus control 0.16±0.04 ng/ml, not significant (n.s)), and normal FSH levels (*Magel2*-null 32.8±2.6 ng/ml versus control 36.8±3.2 ng/ml, n.s).

3.3.4 Reproductive histology is normal in Magel2-null males but age-related changes are present in ovaries of Magel2-null female mice

To determine whether low testosterone levels were associated with histological changes in the testes or impaired spermatogenesis, we compared the

testes of control and *Magel2*-null males. At both 10 and 26 weeks of age there was no difference in the weights of the testes between genotypes, and the architecture of the testes and epididymus were histologically normal in *Magel2*-null males (Figure 3.2). The quantity, morphology, and motility of sperm recovered from the epididymus at both ages were also normal, suggesting that despite reduced testosterone, the male reproductive organs develop and function sufficiently to produce a normal number and quality of gametes.

Reduced reproductive rates in the female mice could be explained by a suboptimal uterine environment, early pregnancy failure, impaired folliculogenesis, missed ovulations, or a combination of these events. There was no difference in the gross anatomy or weight of ovaries and uteri collected from *Magel2*-null females at 26 weeks of age. Because female *Magel2*-null mice that were positive for the presence of a vaginal plug had litters at the same frequency as wild-type, and litter size was only slightly reduced, it is unlikely that *Magel2*-null females have any uterine changes or early pregnancy losses that could explain their reproductive failure. To examine folliculogenesis, diestrus ovaries from 10- and 24-week *Magel2*-null and control females were collected, and examined for the presence and quantity of developing follicles and corpora lutea. At 10 weeks of age, there was no histological difference between *Magel2*-null and control ovaries, with comparable numbers of Graafian follicles and corpora lutea present (Figure 3.3 A–B). In contrast, 26-week old *Magel2*-null females showed an absence of corpora lutea in 10/14 ovaries collected, despite having normal numbers of developing and mature follicles, indicating normal

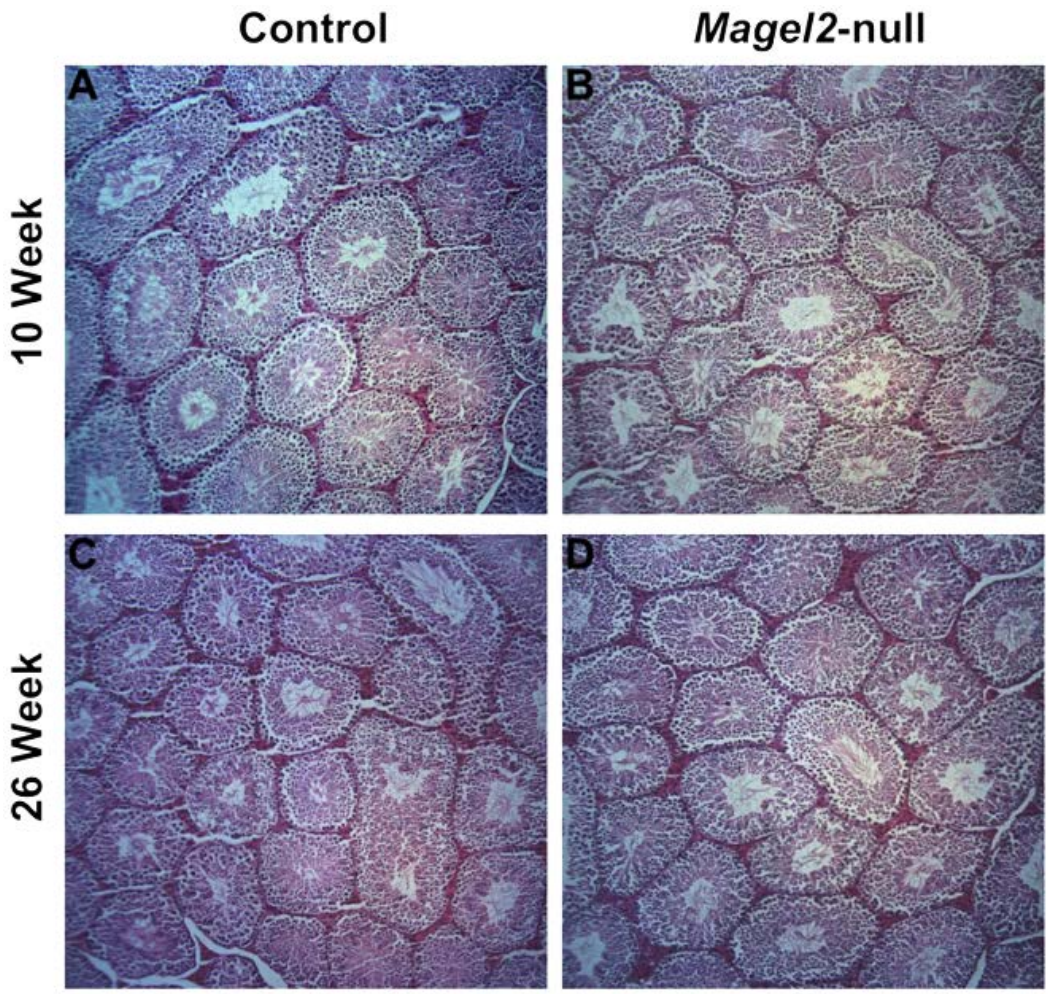


Figure 3.2 Testicular histology in Magel2-null males.

(A, C) Hematoxylin and eosin stained paraffin sections of control testes from 10 **(A)** and 24 week old **(C)** males. **(B, D)** Sections from *Magel2*-null males at both 10 **(B)** and 24 weeks **(D)** show no difference from control sections.

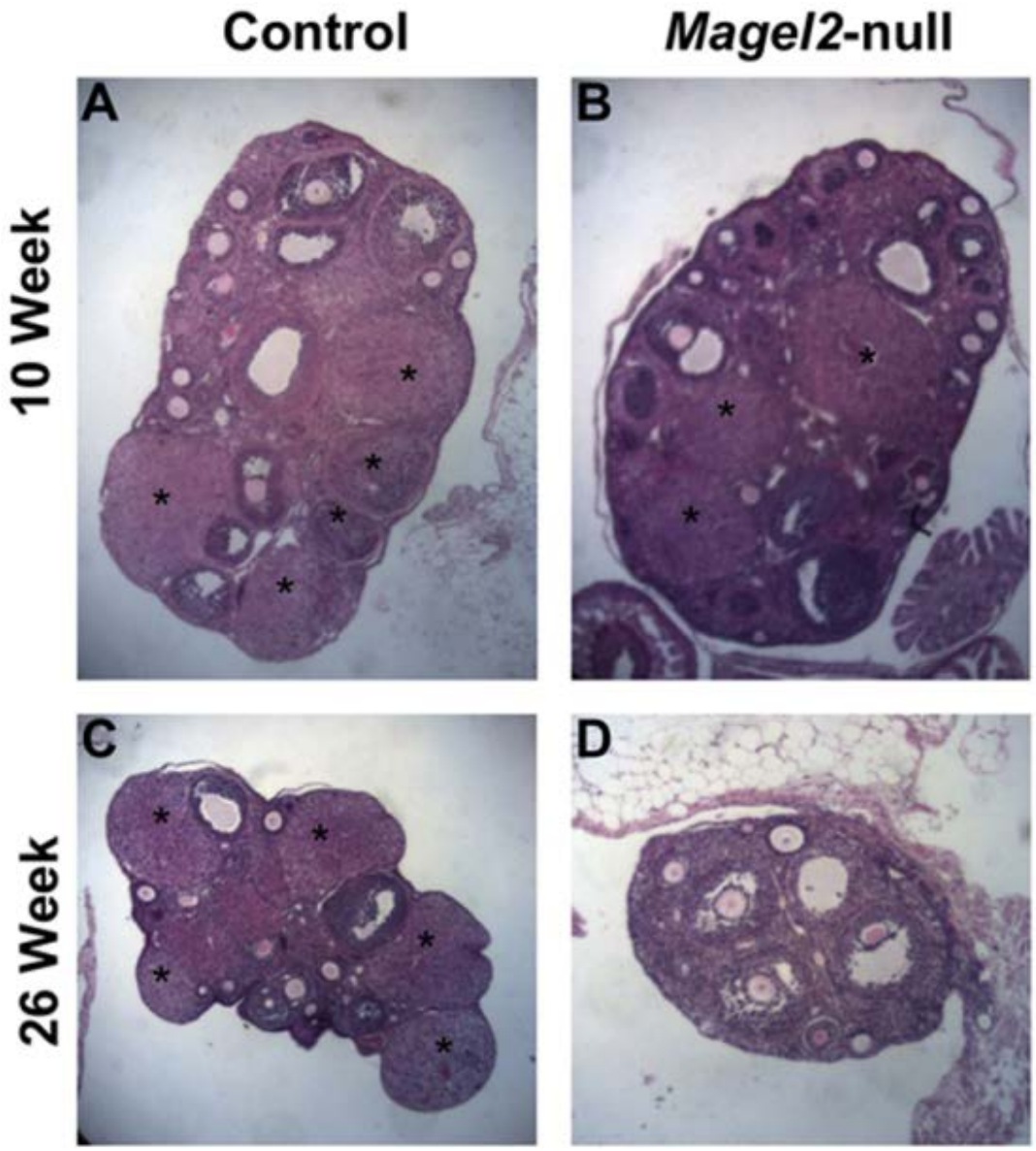


Figure 3.3 Ovarian histology in Magel2-null females.

(A, C) Hematoxylin and eosin stained paraffin sections of control ovaries from 10 **(A)** and 24 week old **(C)** females. **(B, D)** Sections from *Magel2*-null females at 10 weeks **(B)** show no difference from control sections, while sections from 24 week females are notably lacking corpora lutea (indicated by *)**(D)**.

folliculogenesis with missed ovulations in most *Magel2*-null females (Figure 3.3 C–D).

3.3.5 Magel2-null females have abnormal estrous cycles that worsen with age

A normal mouse estrous cycle is defined as 4–5 days in length, with two days in diestrus, one day in proestrus, and one to two days in estrus (Goldman *et al.*, 2007). To determine the reason for extended mating intervals in the female mice, we analyzed the regularity of their estrous cycles. Cyclicity was examined by daily sampling of the vaginal epithelium starting at 8 weeks of age for a minimum of 35 days. The stages of estrous can be easily distinguished cytologically, with nucleated smears during proestrus, cornified smears during estrus, and leukocytic smears during diestrus. Representative profiles from individual female mice are shown in Figure 3.4. The control mice consistently had 4–5 day cycles, with 2 leukocytic smears followed by one nucleated smear and one to two cornified smears (Figure 3.4 A–B). In contrast, the *Magel2*-null mice had abnormal and extended estrous cycles, with few nucleated smears, and lengthened periods with only cornified smears (Figure 3.4 C–D). An additional set of 26 week-old *Magel2*-null and control females were also monitored for estrous cyclicity over a 21 day period, and though the control females still displayed regular cycles (Figure 3.4 E–F), the *Magel2*-null females had even more disruption in their cycling pattern, with only 25% experiencing proestrus (Figure 3.4 G–H). This type of abnormal cycling is similar to that detected in female mice defective in the *Clock* circadian rhythm protein (Miller *et al.*, 2004), and is

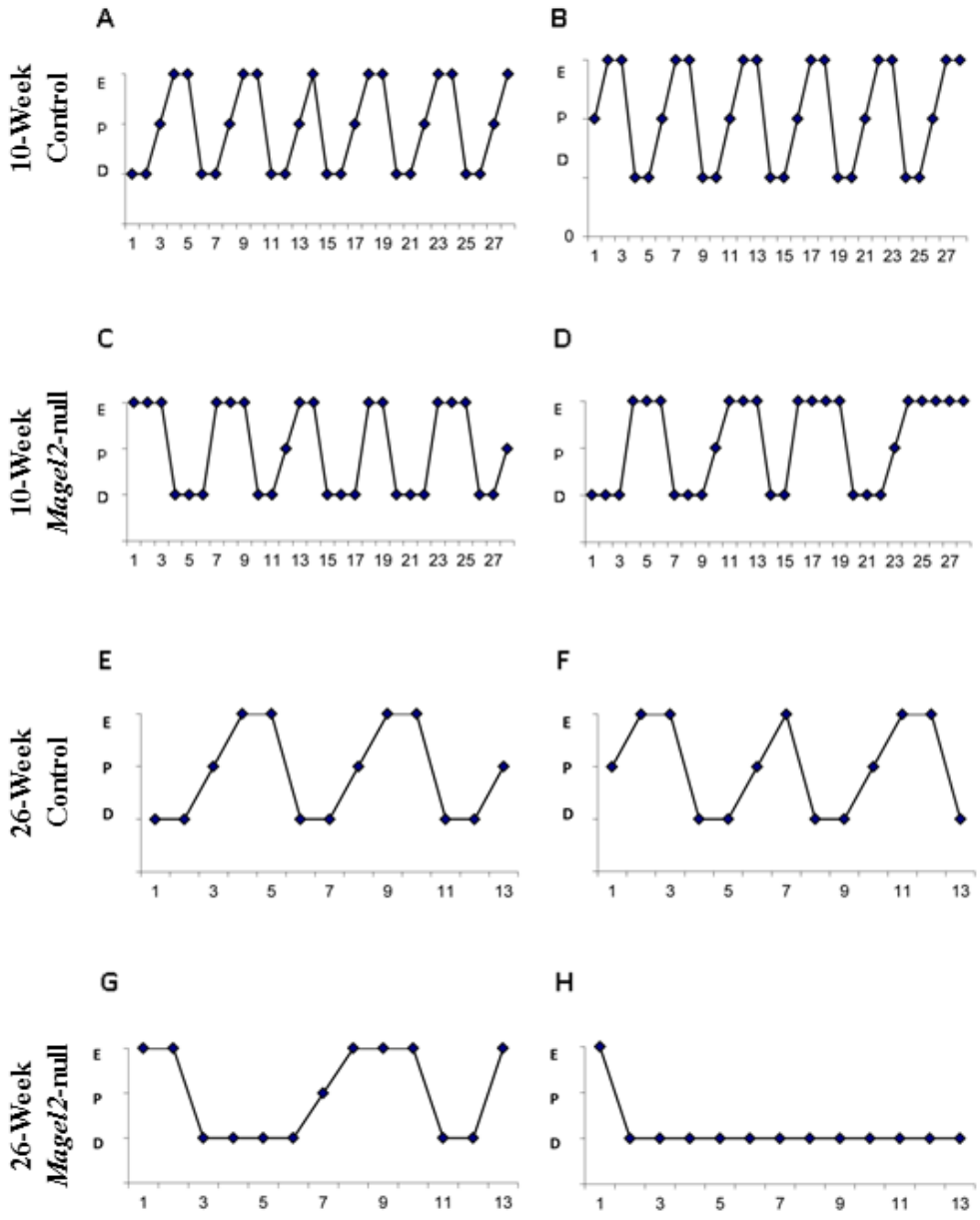

A
B
C
D
E
F
G
H
10-Week Control
10-Week Mageℓ2-null
26-Week Control
26-Week Mageℓ2-null
E
P
D
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27
1 3 5 7 9 11 13

***Figure 3.4 Magel2-null females display longer and irregular estrous cycles determined by vaginal cytology*.**

Representative examples of estrous cycling patterns from two individual female mice of each genotype are shown for each of the two ages tested. **(A–B)** 10 week old control females display regular 4–5 day cycles while 10 week old *Magel*-2 null females spend longer periods in estrus and diestrus, and experience fewer proestrus events **(C–D)**. At 26 weeks of age, control females still display regular cycles **(E–F)** while *Magel2*-null animals have further deteriorated cycling patterns (G–H). The X-axis represents individual days of examination. The Y-axis represents estrus stage: E, Estrus; D, Diestrus; P, Proestrus.

consistent with increased time to mating, reduced fertility with age, and fewer corpora lutea as the ovulatory proestrus period is missed with increased frequency as the female mice age.

3.3.6 Male Magel2-null mice have reduced olfactory preference for estrous female odor

Olfaction is essential for appropriate reproductive behaviour in both sexes, and mice with deficient olfaction can also have reproductive deficits (Yoon *et al.*, 2005). To determine if olfaction is affected in *Magel2*-null mice, we first tested eight male and female mice of each genotype for their ability to find buried food after a 24 h fast. Although 10-week old *Magel2*-null males and females showed no difference in latency to find the buried food compared to control littermates (Figure 3.5A), at 24 weeks of age both the male and female *Magel2*-null mice took significantly longer to locate the buried food than control mice (Figure 3.5B). We then measured the length of time that fasted male mice spent investigating a dried vanilla spot painted on the inside of the cage, during a five minute test period. The *Magel2*-null mice spent only 0.75 s investigating the vanilla spot, far less than the mean time of 6.7 s for the control mice ($p<0.001$, n = 8 mice of each genotype).

To investigate if this apparent olfactory defect affects mating behaviour, we tested olfactory preference in 24 week old male mice by monitoring the time mice spent investigating one of three choices of bedding: 1) bedding soiled by sexually experienced male mice, 2) bedding soiled by female mice in estrus, or 3) unsoiled, clean bedding. We first compared the time spent with soiled bedding

(percent of time spent with soiled bedding, sum of choices 1 and 2) to the time spent with clean bedding. Both control and *Magel2*-null males had a clear preference for soiled bedding, spending 82% of their time with soiled bedding and only 17% with clean bedding (Figure 3.5C).

We then compared the ratio of time spent with bedding soiled by a sexually experienced male versus bedding soiled by an estrus female (choices 1 and 2). Control males showed a clear preference for female-soiled bedding over male-soiled bedding. In contrast, the *Magel2*-null males showed no preference for either male-soiled or female-soiled bedding, spending a similar percentage of their investigation time with either of these two choices (Figure 3.5C).

We conclude that loss of *Magel2* causes reduced reproductive fitness in both male and female mice, with early-onset decline in fertility, irregular estrus cycles in females, and altered olfactory preference in males.

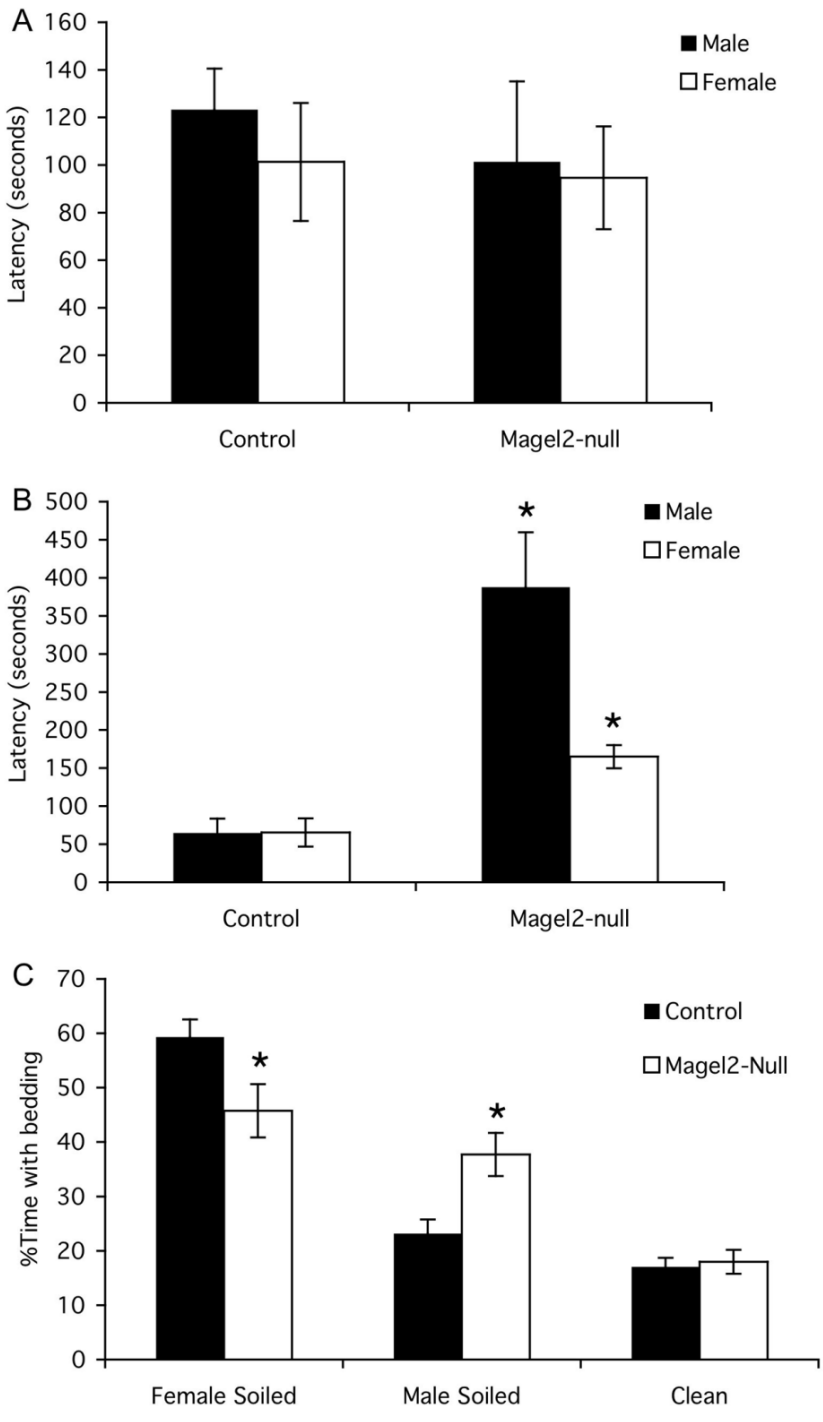

A
Latency (seconds)
160
140
120
100
80
60
40
20
0
Male
Female
Control
Magel2-null
B
Latency (seconds)
500
450
400
350
300
250
200
150
100
50
0
*
*
Male
Female
Control
Magel2-null
C
%Time with bedding
70
60
50
40
30
20
10
0
*
*
Control
Magel2-Null
Female Soiled
Male Soiled
Clean

Figure 3.5 Altered olfaction in Magel2-null mice.

(A) Latency to find buried food tested in 10-week old male mice. **(B)** 24 week-old male *Magel2*-null mice have increased latency to locate the food following a fast in the buried food olfaction test. **(C)** Three way olfactory preference test. For both genotypes, the percent time spent with clean bedding is less than the percent time with soiled bedding (i.e. the sum of the percent time spent with female-soiled bedding and the percent time spent with male-soiled bedding). However, within the time spent with the two soiled bedding choices, control males prefer female-soiled bedding, while *Magel2*-null males spend an equivalent amount of time with the male-soiled bedding as they do with the female-soiled bedding. * $p<0.05$ (control vs. *Magel2*-null), error bars represent SEM.

3.4 Discussion

The aim of these studies was to observe reproductive changes in mice following the loss of the PWS candidate gene *Magel2*, which had previously been shown to cause hypothalamic dysfunction in the areas of circadian function (Kozlov *et al.*, 2007) and metabolism (Bischof *et al.*, 2007). We now show that loss of *Magel2* affects both male and female reproduction, causing reduced fertility and early infertility in both males and females, delayed puberty and irregular estrus cycles in females, and low testosterone, and impaired olfaction and olfactory preference in males.

Young *Magel2*-null mice of both sexes are able to breed, producing relatively normal litters. Some reduced fertility in male mice with only preliminary investigation in female mice was previously reported in *Magel2*-null mice (Kozlov *et al.*, 2007). Normal C57BL/6 mice show an age-related decline in fertility beginning at about 6 months of age, with acyclicity in females beginning around 12 months of age (Goldman *et al.*, 2007). This decline is typically associated with histological changes including vacuolization of seminiferous tubules and increased Leydig cell number in males (Takano and Abe, 1987) and overall loss of follicles and a reduction in developing follicles in females (Gosden *et al.*, 1983). Because the early loss of fertility seen in our mice was not accompanied by these histological changes in the gonads, it does not seem that loss of reproductive ability is linked to accelerating aging in the testes or ovaries in mice lacking *Magel2*.

Magel2-null females have a delay in the onset of puberty, contrasting with the *ClockΔ19* females that display normal timing of both vaginal opening and vaginal estrus (Miller *et al.*, 2004). The onset of puberty in other circadian mutant mice has not been described, though an isolated report suggests a slight delay in puberty in *Bmal1* knockout mice (Boden and Kennaway, 2005). We have previously reported an early failure to thrive in *Magel2*-null neonates followed by adult-onset weight gain and increased adiposity (Bischof *et al.*, 2007), but this weight difference is no longer apparent at the normal timing of puberty. Intriguingly, it has been shown that protein restriction during neonatal development can impair adult fertility by delaying puberty and accelerating reproductive decline in female rats (Guzman *et al.*, 2006), so it is possible that the early failure-to-thrive seen in *Magel2*-null pups contributes to both their delay in puberty and their later reproductive difficulties, but is unlikely to be the sole cause of infertility.

Abnormal estrous cycles in rodents can result from perturbations at any level of the hypothalamic-pituitary-gonadal axis. Because *Magel2*-null females show no anatomical differences of external or internal reproductive organs, and their ovaries have grossly normal folliculogenesis with mature Graafian follicles, ovarian steroidogenesis is not likely perturbed and is therefore not a likely culprit for abnormal estrous cycling in these mice. Rising levels of estrogens produced by developing follicles in the ovary feed back to the hypothalamus, amplifying GnRH pulses, and to the pituitary, sensitizing it to increasing GnRH. The combination of these hypothalamic and pituitary effects result in a precisely timed

surge of LH on the afternoon of proestrus, which leads to ovulation some 10–12 h later (Goldman *et al.*, 2007). Cytological examination of the vaginal epithelium revealed few proestrus events in *Magel2*-null females, coupled with reduced or no corpora lutea in the ovary suggestive of missed ovulation. Normal folliculogenesis in the female *Magel2*-null mice, and normal pituitary hormone levels (LH, FSH) in *Magel2*-null males, suggests a defect in the hypothalamic response to rising estrogen levels, perturbing the precision of the reproductive system. Poor responsiveness could occur despite normal numbers and placement of GnRH neurons. Abnormal estrous profiles and differential ovulation rates have consistently been shown in the *ClockΔ19* mice (Miller *et al.*, 2004, Kennaway *et al.*, 2004, Dolatshad *et al.*, 2006), which do not show a LH surge following proestrus (Miller *et al.*, 2004). Like the *Magel2*-null females we describe here, the *ClockΔ19* mice also have an approximate one pup decrease in litter size, which has been attributed to a reduction in the number of ova per ovulation (Kennaway *et al.*, 2004), but how these ovulations are occurring in the absence of an LH surge remains unexplained.

By the time male *Magel2*-null mice become functionally infertile around the age of 24 weeks, they have significantly lower testosterone levels than controls. Testosterone is produced by Leydig cells in the testes following stimulation by LH pulses from the pituitary gland, which are regulated by rhythmic pulses of GnRH controlled by the SCN. Low testosterone and infertility have been reported in mice lacking *Bmal1*, a core circadian gene also expressed in Leydig cells. These mice have a threefold increase in serum LH, which indicates a

defect in the ability of Leydig cells to produce testosterone (Alvarez *et al.*, 2008). *Magel2* is not expressed in adult mouse testes (Boccaccio *et al.*, 1999), and we did not detect *Magel2*-LacZ reporter gene expression in Leydig cells in adult *Magel2*-null mice. Serum LH levels were not significantly different in the *Magel2*-null males compared to controls, which indicates impaired hypothalamic or pituitary response to the reduced testosterone seen in these mice. Because the structure of the GnRH system appears intact in *Magel2*-null mice, and no testicular abnormalities were detected, abnormal regulation between the central and peripheral components of the hypothalamic-pituitary-gonadal axis is a possible explanation for reduced testosterone levels in the *Magel2*-null mice.

Sexual motivation and successful reproduction in rodents is highly dependent on olfactory ability, with anosmic mice failing to breed (Yoon *et al.*, 2005). Normal male rodents also show a clear preference for estrus female odors compared to male odors, and rats that do not display an olfactory preference for females generally do not copulate (Portillo and Paredes, 2004). *Magel2*-null mice of both sexes develop an olfactory defect between 10 and 24 weeks of age as evidenced by increased time to find food following a fast. Because the null mice also showed reduced foraging and digging during these tests, and because they are generally hypoactive (Kozlov *et al.*, 2007, Bischof *et al.*, 2007)the increased time cannot specifically be attributed to an olfactory defect, and may partially result from decreased food-motivation. Consistent with this idea, during the olfactory preference tests, *Magel2*-null mice clearly preferred soiled bedding over clean bedding, indicating functional olfactory detection. However, while control male

mice prefer estrus female odor, *Magel2*-null mice did not discriminate between bedding soiled by males or soiled by females, indicating a defect in the detection or response to pheromone cues. Pheromone detection in the mouse has been shown to be a result of a direct neural connection between the main olfactory epithelium and the hypothalamic GnRH neuron system (Yoon *et al.*, 2005, Mandiyan *et al.*, 2005), warranting additional examination of the olfactory system in *Magel2*-null mice. Low sexual motivation resulting from reduced testosterone and an inability to respond to female odor cues is the most likely explanation for impaired reproductive function in *Magel2*-null males.

The reduced fertility of *Magel2*-null mice is consistent with that seen in other circadian mutant mice, but subtler than the infertility and incomplete sexual maturation seen in PWS. Delayed and/or partial puberty is often observed in girls with PWS, with late-onset or absent menarche, and amenorrhea or oligomenorrhea in most cases (Burman *et al.*, 2001, Crino *et al.*, 2003). Occasional cases of premature pubarche and true precocious puberty have been described in a proportion of PWS individuals, usually occurring secondary to obesity (Crino *et al.*, 2003). Males with Prader-Willi syndrome typically have hypogonadism, and display Sertoli-only histology in their seminiferous tubules (Eiholzer *et al.*, 2006, Hamilton *et al.*, 1972, Katcher *et al.*, 1977). A recent study of hormone levels in people with PWS aged 16 years and older demonstrated testosterone levels below the normative range in 19 of 23 males, and LH levels below the normative range in 5 of 24 males, while FSH levels were variably low, normal, or high in the same cohort (Brandau *et al.*, 2008). Because several genes

are lost in PWS, combined loss of *Magel2* and other Prader-Willi candidate genes may have an additive deleterious effect. For example, mice lacking a different PWS gene encoding a second MAGE protein, necdin, show a reduction in GnRH neuron number in the hypothalamus (Miller *et al.*, 2008, Muscatelli *et al.*, 2000). Loss of GnRH neuron number, consequent to loss of necdin, coupled with loss of proper feedback circuitry from loss of MAGEL2, would be predicted to have a more serious effect on reproductive function in individuals with PWS with congenital absence of both genes. Further, failure to thrive associated with loss of the snoRNA HBII-85 could contribute to later infertility in PWS, although fertility was reportedly normal in two independent strains of mice deficient for the murine ortholog MBII-85/Snord116 (Ding *et al.*, 2008, Skryabin *et al.*, 2007).

Further studies on reproductive and other hypothalamic functions in *Magel2*-null mice under conditions of worsened (e.g. through exposure to constant darkness) or improved (e.g. by pharmacological reinforcement of the circadian period or more stringent photoperiods) circadian function may provide compelling evidence for additional examination and regulation of circadian rhythm in people with PWS. Our results further highlight the role of *Magel2* in normal hypothalamic function, and add additional support for the role of circadian rhythm in reproduction.

3.5 Bibliography

Alvarez,J. D., Hansen,A., Ord,T., *et al.* (2008) "The circadian clock protein BMAL1 is necessary for fertility and proper testosterone production in mice." J.Biol.Rhythms **23**(1):26-36.

Asnicar,M. A., Koster,A., Heiman,M. L., *et al.* (2002) "Vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating peptide receptor 2 deficiency in mice results in growth retardation and increased basal metabolic rate." Endocrinology **143**(10):3994-4006.

Bischof,J. M., Stewart,C. L. and Wevrick,R. (2007) "Inactivation of the mouse Magel2 gene results in growth abnormalities similar to Prader-Willi syndrome." Hum.Mol.Genet. **16**(22):2713-2719.

Boccaccio,I., Glatt-Deeley,H., Watrin,F., Roeckel,N., Lalande,M. and Muscatelli,F. (1999) "The human MAGEL2 gene and its mouse homologue are paternally expressed and mapped to the Prader-Willi region." Hum.Mol.Genet. **8**(13):2497-2505.

Boden,M. J. and Kennaway,D. J. (2006) "Circadian rhythms and reproduction." Reproduction **132**(3):379-392.

Boden,M. J. and Kennaway,D. J. (2005) "297. Reproduction in the arrhythmic Bmal1 knockout mouse." Reprod.Fertil.Dev. **17**(9):126-126.

Brandau,D. T., Theodoro,M., Garg,U. and Butler,M. G. (2008) "Follicle stimulating and leutinizing hormones, estradiol and testosterone in Prader-Willi syndrome." Am.J.Med.Genet.A. **146A**(5):665-669.

Burman,P., Ritzen,E. M. and Lindgren,A. C. (2001) "Endocrine dysfunction in Prader-Willi syndrome: a review with special reference to GH." Endocr.Rev. **22**(6):787-799.

Champlin,A. K., Dorr,D. L. and Gates,A. H. (1973) "Determining the stage of the estrous cycle in the mouse by the appearance of the vagina." Biol.Reprod. **8**(4):491-494.

Crino,A., Schiaffini,R., Ciampalini,P., *et al.* (2003) "Hypogonadism and pubertal development in Prader-Willi syndrome." Eur.J.Pediatr. **162**(5):327-333.

Ding,F., Li,H. H., Zhang,S., *et al.* (2008) "SnoRNA Snord116 (Pwcr1/MBII-85) deletion causes growth deficiency and hyperphagia in mice." PLoS One **3**(3):e1709.

Dolatshad,H., Campbell,E. A., O'Hara,L., Maywood,E. S., Hastings,M. H. and Johnson,M. H. (2006) "Developmental and reproductive performance in circadian mutant mice." Hum.Reprod. **21**(1):68-79.

Eiholzer,U., l'Allemand,D., Rousson,V., *et al.* (2006) "Hypothalamic and gonadal components of hypogonadism in boys with Prader-Labhart- Willi syndrome." J.Clin.Endocrinol.Metab. **91**(3):892-898.

Goldman,J. M., Murr,A. S. and Cooper,R. L. (2007) "The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies." Birth Defects Res.B.Dev.Reprod.Toxicol. **80**(2):84-97.

Gosden,R. G., Laing,S. C., Felicio,L. S., Nelson,J. F. and Finch,C. E. (1983) "Imminent oocyte exhaustion and reduced follicular recruitment mark the transition to acyclicity in aging C57BL/6J mice." Biol.Reprod. **28**(2):255-260.

Gunay-Aygun,M., Schwartz,S., Heeger,S., O'Riordan,M. A. and Cassidy,S. B. (2001) "The changing purpose of Prader-Willi syndrome clinical diagnostic criteria and proposed revised criteria." Pediatrics **108**(5):E92.

Guzman,C., Cabrera,R., Cardenas,M., Larrea,F., Nathanielsz,P. W. and Zambrano,E. (2006) "Protein restriction during fetal and neonatal development in the rat alters reproductive function and accelerates reproductive ageing in female progeny." J.Physiol. **572**(Pt 1):97-108.

Hamilton,C. R.,Jr, Scully,R. E. and Kliman,B. (1972) "Hypogonadotropinism in Prader-Willi syndrome. Induction of puberty and sperm altogenesis by clomiphene citrate." Am.J.Med. **52**(3):322-329.

Katcher,M. L., Bargman,G. J., Gilbert,E. F. and Opitz,J. M. (1977) "Absence of spermatogonia in the Prader-Willi syndrome." Eur.J.Pediatr. **124**(4):257-260.

Kennaway,D. J., Boden,M. J. and Voultsios,A. (2004) "Reproductive performance in female Clock Delta19 mutant mice." Reprod.Fertil.Dev. **16**(8):801-810.

Kennedy,G. C. and Mitra,J. (1963) "Body weight and food intake as initiating factors for puberty in the rat." J.Physiol. **166**:408-418.

Kozlov,S. V., Bogenpohl,J. W., Howell,M. P., *et al.* (2007) "The imprinted gene Magel2 regulates normal circadian output." Nat.Genet. **39**(10):1266-1272.

Lee,S., Walker,C. L. and Wevrick,R. (2003) "Prader-Willi syndrome transcripts are expressed in phenotypically significant regions of the developing mouse brain." Gene Expr.Patterns **3**(5):599-609.

Lee,S., Kozlov,S., Hernandez,L., *et al.* (2000) "Expression and imprinting of MAGEL2 suggest a role in Prader-willi syndrome and the homologous murine imprinting phenotype." Hum.Mol.Genet. **9**(12):1813-1819.

Mandiyan,V. S., Coats,J. K. and Shah,N. M. (2005) "Deficits in sexual and aggressive behaviors in Cnga2 mutant mice." Nat.Neurosci. **8**(12):1660-1662.

Mercer,R. E., Kwolek,E. M., Bischof,J. M., van Eede,M., Henkelman,R. M. and Wevrick,R. (2009) "Regionally reduced brain volume, altered serotonin neurochemistry, and abnormal behavior in mice null for the circadian rhythm output gene Magel2." Am.J.Med.Genet.B.Neuropsychiatr.Genet. **150B**(8):1085-1099.

Miller,B. H., Olson,S. L., Turek,F. W., Levine,J. E., Horton,T. H. and Takahashi,J. S. (2004) "Circadian clock mutation disrupts estrous cyclicity and maintenance of pregnancy." Curr.Biol. **14**(15):1367-1373.

Miller,N. L., Wevrick,R. and Mellon,P. L. (2008) "Necdin, a Prader-Willi Syndrome candidate gene, regulates gonadotropin-releasing hormone neurons during development." Hum.Mol.Genet.

Muscatelli,F., Abrous,D. N., Massacrier,A., *et al.* (2000) "Disruption of the mouse Necdin gene results in hypothalamic and behavioral alterations reminiscent of the human Prader-Willi syndrome." Hum.Mol.Genet. **9**(20):3101-3110.

Portillo,W. and Paredes,R. G. (2004) "Sexual incentive motivation, olfactory preference, and activation of the vomeronasal projection pathway by sexually relevant cues in non-copulating and naive male rats." Horm.Behav. **46**(3):330-340.

Skryabin,B. V., Gubar,L. V., Seeger,B., *et al.* (2007) "Deletion of the MBII-85 snoRNA gene cluster in mice results in postnatal growth retardation." PLoS Genet. **3**(12):e235.

Takano,H. and Abe,K. (1987) "Age-related histologic changes in the adult mouse testis." Arch.Histol.Jpn. **50**(5):533-544.

Yoon,H., Enquist,L. W. and Dulac,C. (2005) "Olfactory inputs to hypothalamic neurons controlling reproduction and fertility." Cell **123**(4):669-682.

Chapter 4. *Magel2* is required for leptin-mediated responses in POMC neurons in mice.

This chapter is a modified version of a manuscript submitted for publication.

Magel2 is required for leptin-mediated responses in POMC in mice. R.E. Mercer, S.D. Michaelson, M.J.S. Chee, T.A. Atallah, R. Wevrick, and W.F. Colmers.

R.E Mercer completed all of the experiments in this chapter. S.D. Michaelson assisted with slide preparation for Figures 3 & 4, assisted with data collection for Figure 6 I,K, and assisted with manuscript preparation. M.J.S. Chee assisted with data collection for Figure 2A, and Figure 6 C,E, and F. T.A. Atallah was a summer student supervised by R.E. Mercer who assisted with data collection and analysis for Figures 3 &4.

4.1 Introduction

Energy balance is regulated in part by the coordinated action of specialized neurons within the hypothalamus of the brain, which sense circulating signals of energy stores such as the adipocyte derived hormone leptin (Ahima and Lazar, 2008). The arcuate nucleus (ARC) is a key hypothalamic region involved in energy balance regulation, and a major site for leptin action. Two distinct populations of ARC neurons expressing either Neuropeptide Y (NPY) and Agouti-related peptide (AgRP) or pro-opiomelanocortin (POMC) have opposing effects on energy balance. NPY and AgRP, *via* different mechanisms, stimulate food intake and reduce energy expenditure, with the overexpression of both leading to obesity (Graham *et al.*, 1997, Ollmann *et al.*, 1997, Tiesjema *et al.*, 2009). In contrast, POMC is processed into several shorter peptides including α-MSH, which reduces food intake and stimulates energy expenditure through melanocortin responsive neurons in the paraventricular nucleus and elsewhere. Mutations that affect processing or lead to loss of expression of the POMC gene also cause obesity in mice and humans (Yaswen *et al.*, 1999, Coll *et al.*, 2005, Smart *et al.*, 2006, Krude *et al.*, 2003, Farooqi *et al.*, 2006, Creemers *et al.*, 2008, Challis *et al.*, 2004).

Impaired hypothalamic regulation of energy balance is found in numerous genetic forms of human obesity, including congenital deficiency of leptin (Montague *et al.*, 1997), leptin receptor mutations (Clement *et al.*, 1998), MC4R melanocortin receptor mutations (Farooqi *et al.*, 2003), and Bardet-Biedl Syndrome (Seo *et al.*, 2009), and may also contribute to the severe hyperphagia

and obesity seen in people with Prader-Willi Syndrome (PWS), the most common syndromic genetic obesity disorder in humans (Cassidy and Driscoll, 2009), (Cassidy., 1997, Gunay-Aygun *et al.*, 2001). People with PWS typically have a loss of function of several contiguous genes, including *MAGEL2*.

We previously showed that gene-targeted mice lacking *Magel2* expression become overweight with increased adiposity as adults (Bischof *et al.*, 2007), and are subfertile with delayed puberty, irregular estrous cycles, and early onset infertility (Mercer and Wevrick, 2009). As obesity and infertility are common in animal models with impaired leptin signaling (Israel and Chua, 2010), we hypothesized that impaired leptin signaling may also underlie these findings in *Magel2*-null mice. We now report that *Magel2*-null mice do indeed display physiological leptin resistance, that leptin resistance precedes the development of increased adiposity, and that leptin-mediated responses in POMC neurons are specifically inactivated in these animals.

4.2 Materials & Methods

Mouse breeding and handling

All animal procedures were approved by the University of Alberta Animal Care and Use Committee in accordance with the guidelines of the Canadian Council on Animal Care. Mice were weaned between 3-4 weeks of age and then group housed 3-5 per cage with food (PicoLab Rodent Diet 5001) and water *ad lib.*, and housed under a 12:12 light:dark cycle.

Magel2-null and GFP reporter mice

Magel2-null mice were previously generated via gene-targeted replacement of the open reading frame of *Magel2* with a lacZ reporter cassette (Kozlov *et al.*, 2007). The *Magel2* mouse colony is maintained on a C57Bl/6 background by breeding heterozygous *Magel2*-m/+p mice carrying a maternally inherited *Magel2-lacZ* knock-in allele with C57Bl/6 male mice to generate heterozygous, functionally wildtype offspring. Heterozygous carrier males were then bred with wildtype females to produce experimental animals. This cross generated *Magel2*+m/-p mice that inherited the gene-targeted allele from their fathers, and which are *Magel2*-null because they lack expression of *Magel2* due to genomic imprinting that silences the maternally inherited allele. *Magel2*+/+ (wildtype) littermates serve as controls. Mice were PCR genotyped from ear notch biopsies using oligonucleotide primers spanning the lacZ transgene, and the *Magel2* open reading frame (NLG37F 5' ATG GCT CCA TCA GGA GAA C; and LACZR2 5' GGG ATA GGT CAC GTT GGT GT). Presence of GFP was identified with oligonucleotide primers targeting the GFP gene sequence (GFPF 5'AAGTTCATCTGCACCACCG; and GFPR 5'TCCTTGAAGAAGATGGTGCG).

In order to identify specific neurons within the hypothalamus that express the signaling form of the leptin receptor (LepRb) we crossed *Magel2*-m/+p carrier males with previously described homozygous $LepRb^{EGFP}$ females (Leshan *et al.*, 2006, Leshan *et al.*, 2009), which express green fluorescent protein in LepRb+ cells. This cross produces *Magel2*x$LepRb^{EGFP}$ mice, lacking expression of *Magel2*

but expressing LepRb^{EGFP}, and control LepRb^{EGFP} littermates carrying a wildtype *Magel2* gene. To identify POMC neurons within the hypothalamus, *Magel2*-m/+p carrier males were crossed with homozygous POMC^{EGFP} females (The Jackson Laboratory stock #009593, Bar Harbor, ME, USA), which express green fluorescent protein under the transcriptional control of POMC genomic sequences (Cowley *et al.*, 2001). This cross produces *Magel2*xPOMC^{EGFP} mice, lacking expression of *Magel2*, and control POMC^{EGFP} littermates that are wildtype for *Magel2*.

Food withdrawal and refeeding

Male adult (12-16 weeks of age) *Magel2*-null and control mice were singly housed for at least one week prior to the beginning of the experiment. At the onset of the study, mice were weighed and then subjected to a 48 h fast beginning at 1600h. Body weight was recorded following 24 h and 48 h of fasting, and food intake and body weight change were measured during refeeding for 3 days. Food intake was measured by weighing food before and after each procedure, taking care to account for food spilled in the bottom of the cage.

Leptin and melanocortin sensitivity assays

Magel2-null and control male mice were singly housed for at least one week before the start of the experiment. One week before leptin injections, all animals were injected every 24 h for 3 days with phosphate buffered saline, pH 7.3 (PBS). Body weight and food intake were measured during this time. On day 1 of the experiment, food was removed from the cages at 1500h. At 1600h, mice were injected intraperitoneally (ip) with either 2.5 mg/kg mouse recombinant

leptin (Dr. A.F. Parlow, National Hormone and Peptide Program, NHPP-NIDDK, Torrance, CA, USA) or PBS. Food was returned to the cage, and food intake measurements were taken 2, 4, 16, and 24 h later. After 3 days recovery, the experiment was repeated with the animals that received PBS in the first round receiving leptin in the second round and *vice versa*. To assess the responsiveness of the melanocortin system, we used the synthetic melanocortin agonist melanotan-II (MT-II, Phoenix Pharmaceuticals, Burlingame, CA, USA), which acts on both MC3 and MC4 receptors (Hruby *et al.*, 1995, Cowley *et al.*, 1999). The melanocortin responsiveness experiment was performed as indicated in the leptin sensitivity assay, only instead of leptin, mice were injected ip with 2.5 mg/kg MT-II.

Leptin stimulation and immunohistochemistry

Adult (12-16 weeks) *Magel2*xPOMC^{EGFP} and control mice of both genders were handled daily for 2 weeks, including one week of PBS injections in order to minimize injection-related c-fos responses in the brain. On the day of sacrifice, mice were injected ip with either 2.5 mg/kg recombinant mouse leptin or PBS 45 min. before being anesthetized with pentobarbital sodium (Euthanyl, Bimeda MTC, Cambridge, ON, Canada). Anesthetized mice were perfused directly into the left ventricle of the heart with 25 ml cold PBS followed by 30 ml of 4% paraformaldehyde. Brains were removed and submerged in 4% paraformaldehyde for 24 h at 4°C, and then cryoprotected in 25% sucrose for an additional 48 h at 4°C before being frozen on dry ice in Shandon Cryomatrix (Thermo Scientific, Pittsburgh, PA, USA) and kept at -80°C until sectioning. Coronal 30 µm sections

of the hypothalamus were cut sequentially on a cryostat and collected as free-floating sections in 4 series, and stored in 0.02% sodium azide in PBS at 4°C until use. For pSTAT3 immunohistochemistry (IHC), sections were pretreated with 1% NaOH and 1% H_20_2 in H_20 for 20 min, 0.3% glycine in PBS for 10 min, and 0.03% sodium dodecyl sulfate in PBS for 10 min. Subsequently, sections were blocked for 1 h with 3% normal goat serum in PBS/0.3% Triton X-100, and then incubated overnight with pSTAT3 antibody (1:1000, 9131 Cell Signaling, Danvers, MA, USA) in PBS/0.3% Triton X-100. The following day, sections were washed with PBS/0.3% Triton X-100 and incubated for 2 h with goat anti-rabbit secondary antibody labeled with Alexa Fluor 594 (Invitrogen, Carlsbad, CA, USA). For c-fos and POMC IHC, sections were washed 3 times in PBS, and then blocked for 30 min. in 3% normal serum in PBS/0.3% Triton X-100. Sections were then incubated for 48 h at 4°C with primary antibody (c-fos (Ab-5), 1:2000, PC-38, Millipore, Billerica, MA, USA; POMC, anti-GFP, 1:4000, ab13970, AbCam). Sections were washed 3 times with PBS/0.3% Triton X-100 and then incubated for 2 h with goat anti-rabbit secondary antibodies labeled with Alexa Fluor 594 or goat anti-chicken secondary antibodies labeled with Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) as appropriate at 1:500 dilution in PBS/0.3% Triton X-100, for 2 h at room temperature, in the dark. Following secondary antibody staining, sections were washed thoroughly with PBS, mounted on slides using ProLong Gold (Invitrogen, Carlsbad, CA, USA), and images captured using a Zeiss LSM510 confocal microscope. For cell counting in the arcuate nucleus, sections were organized in a rostral to caudal manner through

the hypothalamus according to the mouse brain atlas (Rosen *et al.*, 2000). For pSTAT3, cells were counted using MetaMorph Imaging Suite (Molecular Devices, Sunnyvale, CA, USA), and for c-fos and POMC, cells were counted using Image J (National Institutes of Health, Bethesda, MD, USA).

Slice preparation and electrophysiology

Brains from 6-12 week old male and non-estrous female mice carrying a $LepRb^{EGFP}$ or $POMC^{EGFP}$ transgene were rapidly removed and sliced for patch clamp electrophysiology recordings. Slicing and electrophysiology procedures were similar to those previously described (Chee *et al.*, 2010, Chee *et al.*, 2011). Slices were incubated for at least 1 h at room temperature in carbogenated artificial cerebrospinal fluid (aCSF) containing the following (in mM): 124 NaCl, 3 KCl, 1.3 $MgSO_4$, 1.4 NaH_2PO_4, 2.5 glucose, 7.5 sucrose, 26 $NaHCO_3$ and 2.5 $CaCl_2$ (osmolarity 300-305 mOsm/L), prior to each experiment. For electrophysiology, slices were continuously perfused (2-4 ml/min) with warm (32-34°C), carbogenated aCSF. Epifluorescent illumination was briefly used to identify cells expressing green fluorescence, at which time the light source was switched to infrared-differential interference contrast imaging to obtain whole-cell recordings. Thin-walled glass pipettes (World Precision Instruments Inc., Sarasota, FL, USA), pulled from a two-stage microelectrode puller (Narishige Scientific, Japan), had resistances of 5-7 MΩ when backfilled with an internal solution containing (in mM): 126 K-gluconate, 4 KCl, 10 HEPES, 5 MgATP, 0.3 NaGTP, 1 EGTA, 0.3 $CaCl_2$ and 0.02% neurobiotin (pH adjusted to 7.25 with KOH, osmolarity adjusted to 280 mOsm/L). Visualized-patch whole-cell

recordings were obtained from patch pipettes attached to a headstage of an Axoclamp 2A amplifier (Axon Instruments). Electrophysiological recordings in current- and voltage-clamp modes were filtered at 3 kHz and sampled at 5-10 kHz via a Digidata 1322A interface (Molecular Devices) interface connected to a computer running PCLAMP 9.2 software (Molecular Devices). Data were stored and analyzed using a computer equipped with Clampex 10.2 (Molecular Devices). Stock solutions of mouse recombinant leptin were prepared in PBS (pH 7.8), while stock solutions of human NPY (Peptidec Technologies Ltd.,Pierrefonds, QC, Canada) were prepared in HPLC grade water, then stored at -20°C. NPY and leptin were diluted into aCSF from concentrated stock solutions to experimental concentrations immediately before use. These were perfused into the recording chamber by gravity for a minimum of 3 min. After each successive drug application, the slice was continuously washed with aCSF for a minimum of 10 min. before the next addition of drug. A stable and reversible change in membrane potential of at least 2mV from baseline appearing within a few minutes of the onset of drug application was considered a valid pharmacological response.

Statistical analysis

Statistical analyses of differences between genotypes were performed using the Student's unpaired t-test or the Fisher's Exact Test functions of the GraphPad Prism 5 software (GraphPad, La Jolla, CA, USA). Measurements are reported as the mean ± SEM. Differences with $p<0.05$ after correction for multiple t-testing were considered significant, whereas p values greater than 0.05 were considered not significant (n.s.).

4.3 Results

4.3.1 Magel2-null mice lose less weight during a fast, and eat less food and gain less weight after fasting.

Leptin maintains homeostatic control of weight, regulating ingestive behaviour and energy expenditure in response to changes in nutritional energy availability. The fall in circulating leptin that occurs with food deprivation normally causes increased feeding when food is reinstated, restoring normal weight and fat mass (Ahima and Lazar, 2008). However, refeeding-associated weight gain and hyperphagia are dysregulated in mice with diet-induced obesity (Becskei 2009) or mice carrying mutations that selectively ablate POMC neurons (Xu *et al.*, 2005a, Xu *et al.*, 2007) or decrease levels of hypothalamic neuropeptides (Bannon *et al.*, 2000, Segal-Lieberman *et al.*, 2003, Patel *et al.*, 2006). To determine if *Magel2* is important for compensatory responses after fasting, we subjected mice to a prolonged (48 h) fast. While control mice lost 16% of their body weight after 48 h of fasting *Magel2*-null mice lost significantly less body weight (12% of initial weight), consistent with their previously noted reduced locomotor activity (Figure 4.1A). We then refed the fasted mice, and measured food intake and body weight over the next 3 days. Body weight returned to baseline within 2 days of refeeding in control mice, but *Magel2*-null mice remained underweight even after 3 days (Figure 4.1A). Food intake was similar before fasting (Figure 4.1B), but *Magel2*-null mice ate less food during the initial 24 h recovery period (Figure 4.1C), resulting in a significantly reduced food intake ratio compared with control mice

(Figure 4.1D). These results suggest that the hypothalamic pathways required for compensatory refeeding are defective in the *Magel2*-null mice.

4.3.2 Magel2-null mice lack the anorexigenic response to peripherally administered leptin

Magel2-null mice have excess adipose tissue, and high levels of circulating leptin suggesting reduced leptin sensitivity. At 20 weeks of age, *Magel2*-null mice are 14% heavier than control mice (Figure 4.2A). To examine whether *Magel2*-null mice are sensitive to exogenous leptin, we measured food intake in singly housed male mice using a crossover study design in which the same animals received either ip leptin (2.5 mg/kg) or PBS approximately 1 week apart. In control leptin-treated mice, food intake was reduced by about 30% in the 24 h following leptin injection, as expected. However, leptin-treated *Magel2*-null mice showed no reduction in food intake following ip leptin (Figure 4.2B). Excess adiposity can decrease leptin sensitivity even in the absence of a genetic mutation (Van Heek *et al.*, 1997, Munzberg *et al.*, 2004). We therefore repeated this study in younger (6-week old) mice, where there is no difference in body weight between *Magel2*-null and control animals (Figure 4.2C). Leptin treatment in young control mice again caused a reduction of approximately 35% in 24 h food intake compared to PBS treatment. In contrast, there was no reduction in 24 h food intake in leptin-treated young *Magel2*-null mice (Figure 4.2D), so that leptin insensitivity precedes excessive weight gain in *Magel2*-null mice. These results suggest that *Magel2* is required for the anorexigenic effect of leptin in mice.

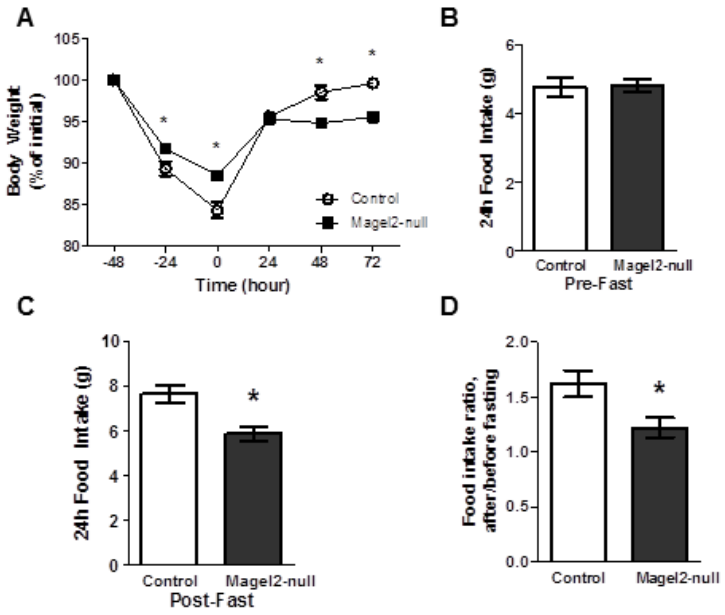


Figure 4.1 Magel2-null mice have abnormal weight recovery and compensatory refeeding after fasting.

A) Body weights of adult male mice subjected to a 48 hour fast and a 72 hour refeeding period. *Magel2*-null mice lost less weight while fasting, and recovered less weight during refeeding. B) 24h food intake pre-fast and C) post-fast. D) *Magel2*-null mice have a reduced food intake ratio compared to controls. Controls, n=6, *Magel2*-null, n=6. Values are means ± SEM. *$p<0.05$, compared by Student's t-test.

4.3.3 Magel2 deficiency reduces leptin-mediated phosphorylation of STAT3 and induction of c-fos expression in the arcuate nucleus

We next examined the activation of the leptin receptor by measuring levels of phosphorylated STAT3 (pSTAT3) (Bjorbaek *et al.*, 1997, Munzberg *et al.*, 2003, Piper *et al.*, 2008), in the ARC following a single ip leptin (2.5 mg/kg) injection. While few pSTAT3 positive neurons were seen in the ARC following PBS injection in both *Magel2*-null and control animals (Figure 4.3A, C, E), numerous pSTAT3 positive cells were seen in the ARC of both genotypes after leptin injection (Figure 4.3B, D). Nonetheless, detailed cell counts throughout the ARC revealed a 30-35% reduction in pSTAT3 positive cells in *Magel2*-null mice compared to control (Figure 4.3E). Next, we measured the induction of c-fos, an immediate early gene marker of neuronal activation that is detected in POMC but not NPY neurons in the ARC after leptin injection (Dragunow and Faull, 1989, Elias *et al.*, 1999).

Baseline c-fos immunoreactivity was observed in PBS-injected control animals (Figure 4.4A, C, E), and leptin treatment caused a significant increase in c-fos expression in both control and *Magel2*-null mice (Figure 4.4B, D, E), particularly in more posterior regions of the ARC where the majority of leptin sensitive POMC neurons are located (Williams *et al.*, 2010). Interestingly, both baseline (PBS) and leptin-induced c-fos positive cells were reduced in number in *Magel2*-null mice compared to control (Figure 4.4E).

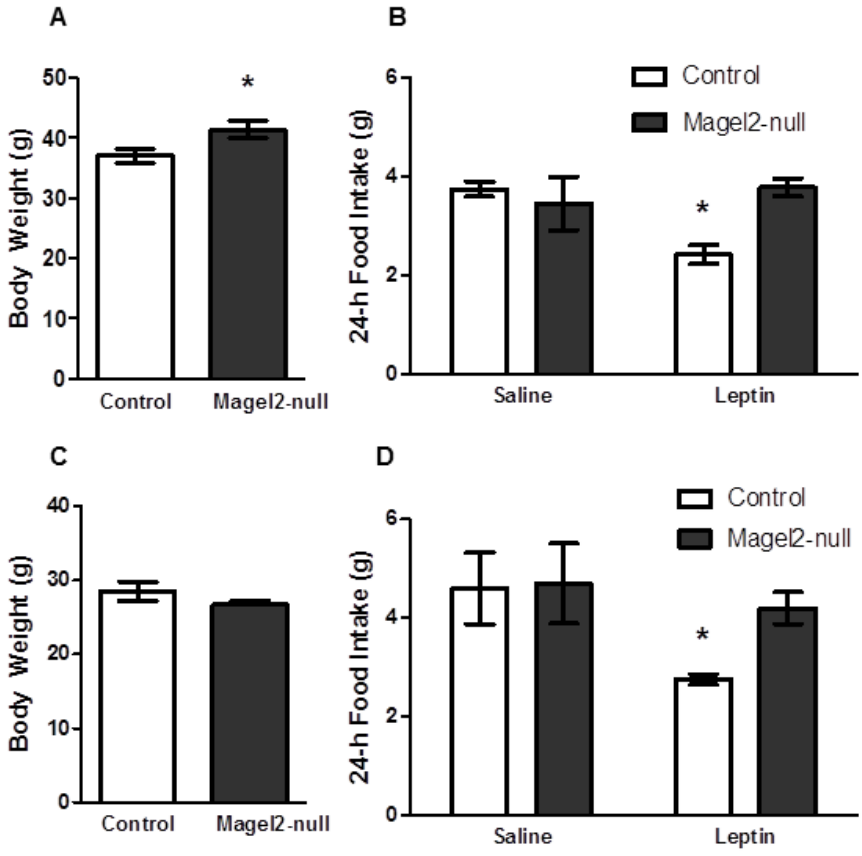
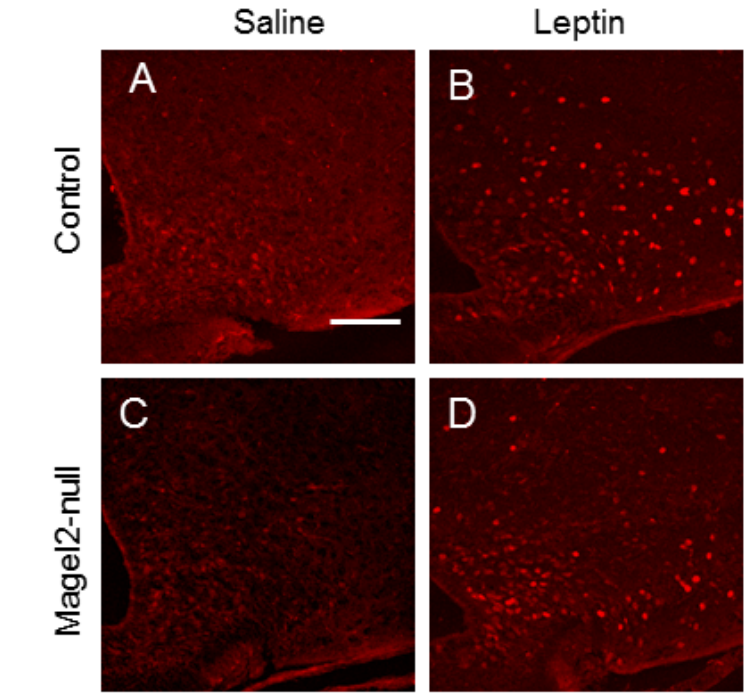

A
B
C
D
Body Weight (g)
24-h Food Intake (g)
Control
Magel2-null
Saline
Leptin
*

Figure 4.2 Peripherally administered leptin fails to reduce food intake in Magel2-null mice.

A) Body weights of 20 week old male mice and B) 24 hour food intake following leptin or PBS (saline) injections. Leptin reduced food intake in control mice, while leptin-treated *Magel2*-null mice showed no significant change in food intake. Controls, n=10, *Magel2*-null, n=10. C) Body weights of 6 week old male mice and D) 24 hour food intake following leptin or PBS injections. Leptin reduced food intake in control but not *Magel2*-null mice. Controls, n=7, *Magel2*-null, n=6. Values are means ± SEM. *$p<0.05$, compared between genotypes by Student's t-test.


Saline
Leptin
A
B
Control
C
D
Magel2-null

E

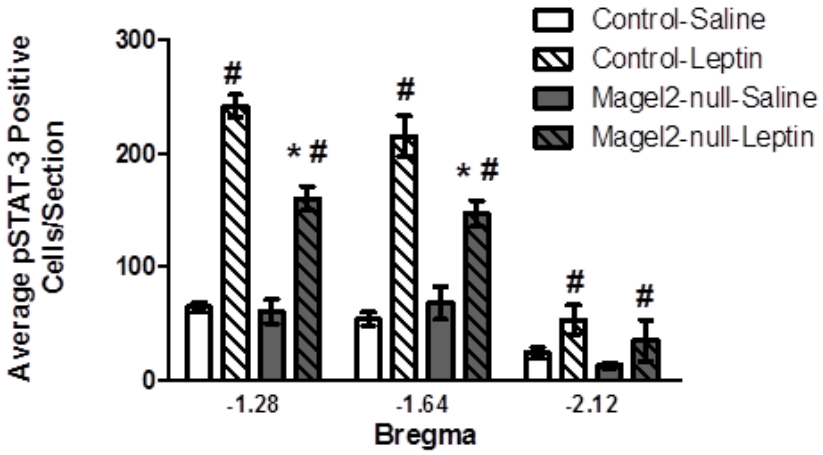

Control-Saline
Control-Leptin
Magel2-null-Saline
Magel2-null-Leptin
Average pSTAT-3 Positive Cells/Section
300
200
100
0
#
* #
-1.28
-1.64
-2.12
Bregma

Figure 4.3 pSTAT3 activation in ARC neurons in leptin-treated mice.

A-D) Representative IHC images showing pSTAT3 immunoreactivity following ip PBS (saline) and leptin injection. Scale Bar 100 μm. E) *Magel2*-null mice have fewer pSTAT3 positive cells following leptin injection. Values are means ± SEM. *p<0.05, *Magel2*-null compared to control, #p<0.05, leptin-injected compared to saline-injected, by Student's t-test.

4.3.4 Magel2-null mice have reduced numbers of ARC POMC neurons

POMC neurons form an important part of the hypothalamic energy balance neurocircuitry, and are activated in response to leptin (Cowley *et al.*, 2001). Fewer leptin-induced pSTAT3 and c-fos immunoreactive cells were observed in the ARC of *Magel2*-null mice, particularly in areas previously shown to contain higher levels of leptin-sensitive POMC neurons. We therefore counted POMC/EGFP positive neurons in the ARC of *Magel2*xPOMC^{EGFP} and control mice, and found on average 39% fewer POMC+ neurons in the *Magel2*-null mice than in controls (Figure 4.5). This reduction was most prominent in the more posterior region of the ARC, where a 52% reduction in POMC positive cells was found (Figure 4.5C). Thus, loss of POMC could partially but not fully explain the reduction seen in leptin-induced pSTAT3 and c-fos expression in the ARC of *Magel2*-null mice.

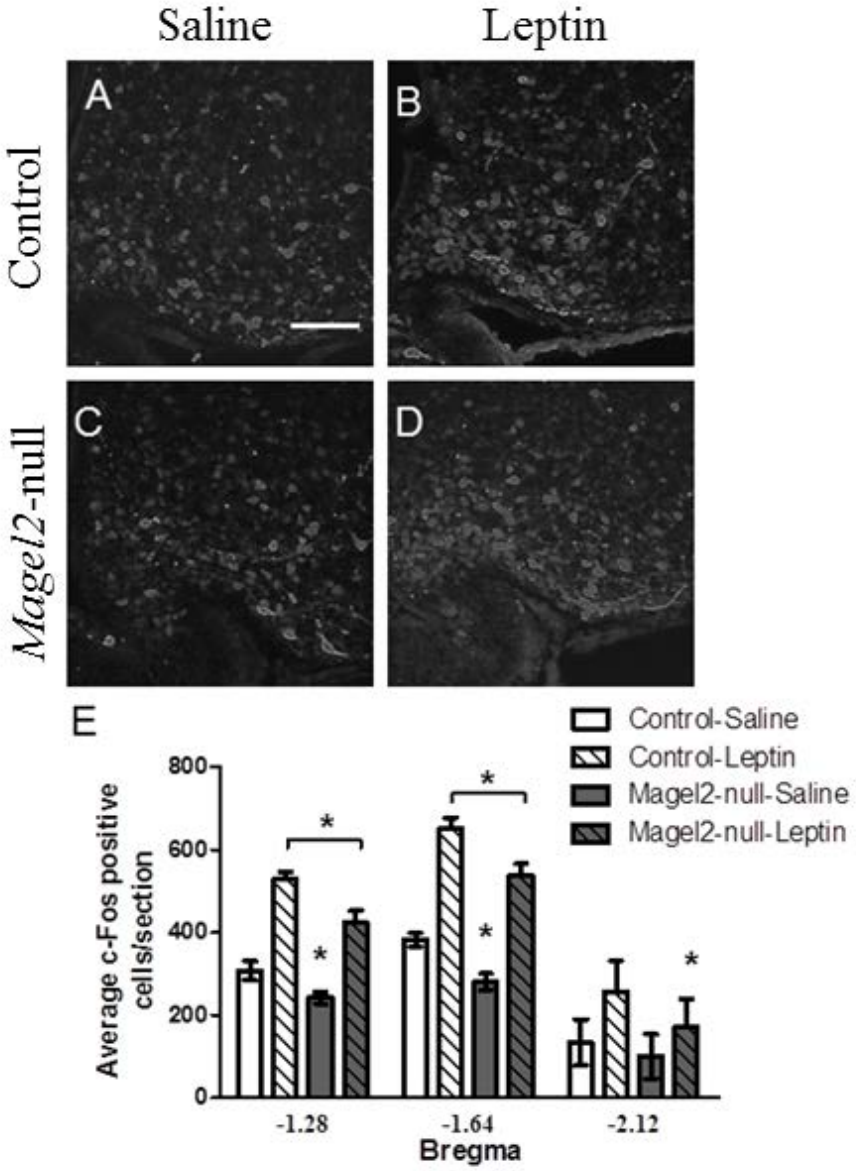

Saline
Leptin
A
B
Control
C
D
Magel2-null
E
Control-Saline
Control-Leptin
Magel2-null-Saline
Magel2-null-Leptin
Average c-Fos positive cells/section
800
600
400
200
0
*
*
*
*
*
-1.28
-1.64
-2.12
Bregma

Figure 4.4 Expression of c-fos in ARC neurons following leptin injection.

A-D) Representative images of c-fos IHC following ip PBS and leptin injection. Scale Bar 100 μm. E) Leptin induced c-fos expression in both *Magel2*-null and control mice compared to PBS. At both baseline and following leptin treatment, *Magel2*-null mice had significantly fewer c-fos positive cells compared to controls. Values are means ± SEM. *$p<0.05$, compared between genotypes by Student's t-test.

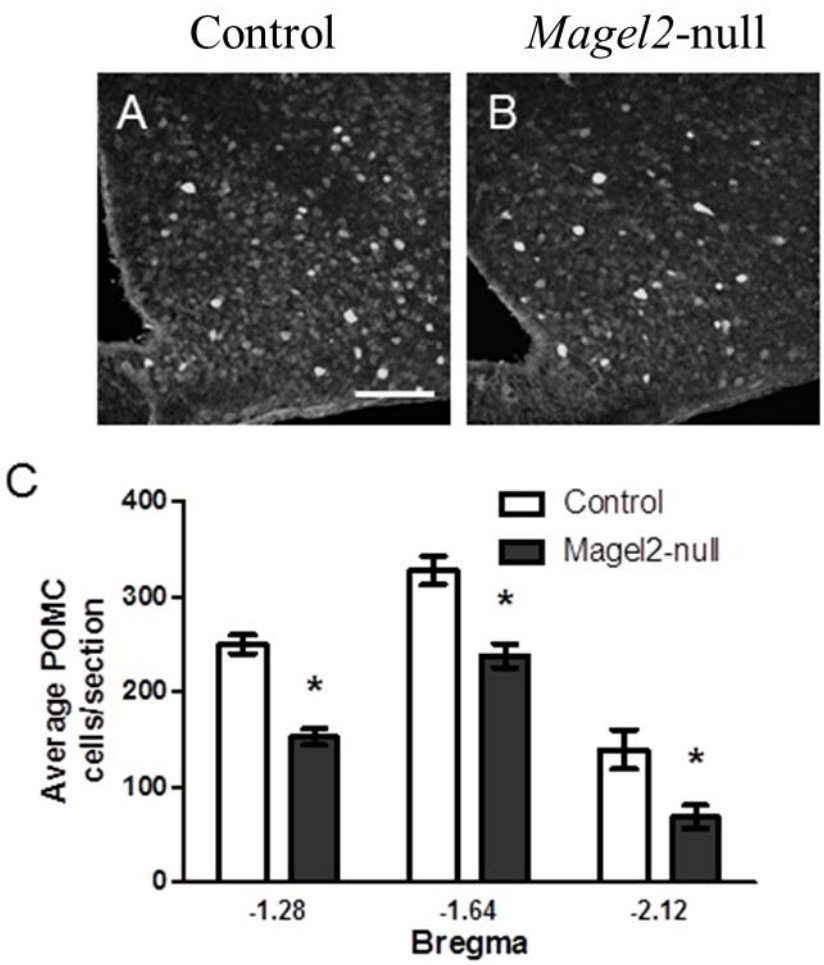


Figure 4.5 Magel2-null mice have fewer ARC POMC neurons.

A-B) Representative images of GFP (POMC) IHC. Scale bar 100 μm. C) *Magel2*-null mice have fewer GFP expressing (POMC) cells at all levels of the ARC (Bregma -1.28, 39% reduction, $*P < 10^{-8}$; Bregma -1.64, 27% reduction, $*P < 10^{-5}$; Bregma -2.12, 52% reduction, $*P < 0.01$), compared between genotypes by Student's *t*-test. Values are means ± SEM.

4.3.5 Leptin fails to activate POMC neurons in the ARC of Magel2-null mice

To directly examine leptin responses in ARC neurons, we performed whole-cell visualized-patch recordings of fluorescent neurons in *Magel2*xLepRb^{EGFP} and control mice (sample cell shown in Figure 4.6 A, B). First, the resting membrane potential (RMP) of LepRb+ neurons (Figure 4.6C) was comparable in the two genotypes. NPY hyperpolarizes the majority of leptin-responsive ARC cells (Figure 4.6D) (Acuna-Goycolea and van den Pol, 2005, Roseberry *et al.*, 2004). Application of either 100 nM or 300 nM NPY produced a robust hyperpolarization in virtually all ARC LepRb+ neurons tested in both *Magel2*-null and control slices, indicating that the loss of *Magel2* does not interfere with normal NPY signaling (Figure 4.6E-F). We then examined the leptin (100 nM) responses in LepRb+ neurons in the ARC. Leptin normally activates (depolarizes) POMC neurons, and inhibits (hyperpolarizes) NPY neurons (Cowley *et al.*, 2001, van den Top *et al.*, 2004), so we expected to observe both responses in the mixed neuronal populations represented by LepRb+ cells in the ARC. Indeed, leptin induced both hyperpolarizing and depolarizing responses in slices from control mice, with a few unresponsive cells. All cells, including leptin-unresponsive cells, exhibited a normal electrophysiological response to 300 nM NPY. In striking contrast, in slices from *Magel2*-null mice, we did not observe neurons with depolarizing responses to leptin. Hyperpolarizing responses were seen at a frequency comparable to controls, while leptin-unresponsive cells (which nevertheless showed normal NPY responses) were present in much higher numbers (Figure 4.6I). These results suggest that the

inhibitory action of leptin is unaffected in ARC LepRb+ neurons of *Magel2*-null mice, but that the excitatory effect of leptin, typically observed at POMC neurons, is selectively absent in *Magel2*-null mice.

To more directly examine the population of neurons specifically activated by leptin in the ARC, we identified POMC neurons using crosses with mice expressing GFP in POMC cells (*Magel2*xPOMC^{EGFP} and littermate controls). As with LepRb+ neurons in the ARC, POMC neurons from control and *Magel2*-null animals did not differ in their RMP (Figure 4.6J). We then tested leptin (100 nM) responses in POMC+ cells located in the posterior and medial regions of the ARC, where a large number of POMC neurons are leptin sensitive (Williams *et al.*, 2010). Leptin induced depolarization in the majority of POMC neurons from control mice, but no depolarizing effects were seen in response to leptin in *Magel2*-null mice. This confirms that POMC neurons in these animals are indeed insensitive to acute administration of leptin (Figure 4.6K).

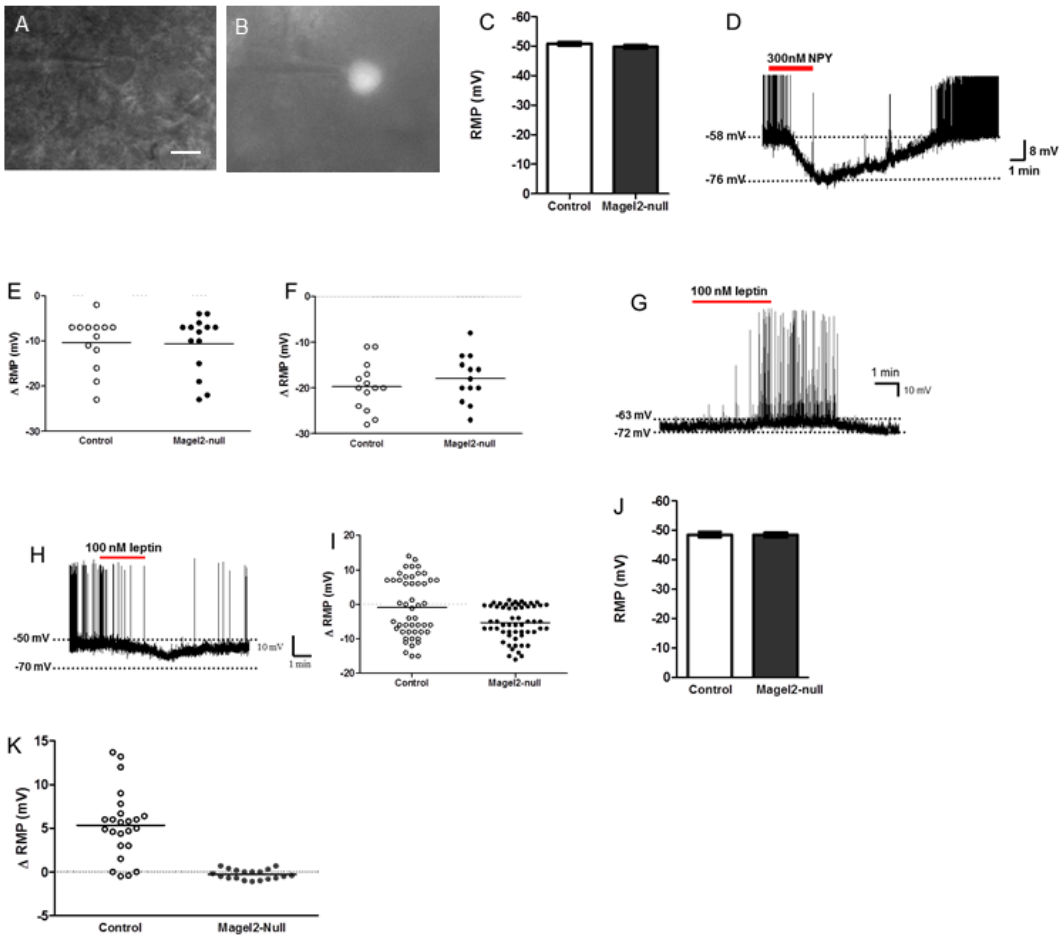

A
B
C
RMP (mV)
Control
Magel2-null
D
300nM NPY
-58 mV
-76 mV
8 mV
1 min
E
Δ RMP (mV)
Control
Magel2-null
F
Δ RMP (mV)
Control
Magel2-null
G
100 nM leptin
1 min
10 mV
-63 mV
-72 mV
H
100 nM leptin
-50 mV
-70 mV
10 mV
1 min
I
Δ RMP (mV)
Control
Magel2-null
J
RMP (mV)
Control
Magel2-null
K
Δ RMP (mV)
Control
Magel2-Null

Figure 4.6 Magel2 is required for the leptin-induced depolarizing response in POMC neurons.

Example of a $LepRb^{EGFP}$ positive neuron identified for electrophysiological recordings using: A) infrared-differential interference contrast and B) epifluorescence imaging. Scale bar, 10 μm. C) Mean RMP of ARC LepRb+ neurons. D) Current clamp recording of a $LepRb^{EGFP}$ neuron showing the hyperpolarizing effect of 300 nM NPY. There was no change in the magnitude of hyperpolarization between control and *Magel2*-null neurons treated with either E) 100 nM or F) 300 nM NPY. G-H) Current clamp recordings of typical responses to 100 nM leptin in G) depolarizing neurons, and H) hyperpolarizing neurons. I) Changes in RMP with application of 100 nM leptin to ARC $LepRb^{EGFP}$ neurons. Circles represent individually tested neurons. Depolarizing, hyperpolarizing, and unresponsive neurons were found in control slices, while only hyperpolarizing and unresponsive neurons were found in *Magel2*-null slices. J) Mean RMP of ARC $POMC^{EGFP}$ neurons. K) Changes in RMP caused by application of 100 nM leptin to ARC $POMC^{EGFP}$ neurons. Depolarizing responses were observed in control but not *Magel2*-null mice. RMP values are means ± SEM. *$p<0.05$, compared between genotypes by Fishers Exact Test.

4.3.6 Magel2-null mice are hypersensitive to the melanocortin agonist MT-II

A failure of POMC neurons to depolarize in response to leptin is predicted to cause loss of release of α-MSH. In other animal models of leptin insensitivity, an enhanced anorexigenic response to α-MSH or the melanocortin agonist MT-II occurs (Enriori *et al.*, 2007, Hansen *et al.*, 2001, Scarpace *et al.*, 2003, Li *et al.*, 2004). We therefore examined the effect of the synthetic melanocortin agonist MT-II on food intake in *Magel2*-null mice. Mice were fasted for 24 hours, then injected with MT-II (2.5 mg/kg ip). Compared to PBS-injected control fasted mice, MT-II injected control mice consumed 50% less food over the first 2 h of refeeding. After this time there was no significant difference in food intake between control mice injected with MT-II or PBS. In contrast, *Magel2*-null mice injected with MT-II had a greater reduction in food intake compared to PBS injection, and this decrease was still evident after 24 h (Figure 4.7). This result suggests that the melanocortin system is chronically upregulated in *Magel2*-null mice, likely as a result of the loss of melanocortinergic tone from ARC POMC neurons.

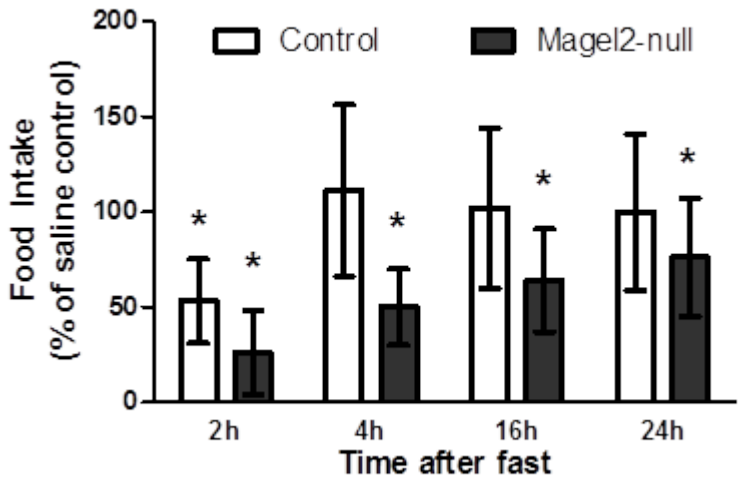


Figure 4.7 Effects of ip MT-II on food intake.

Food intake following 2.5 mg/kg injection of MT-II or saline in 24 h fasted mice. MT-II significantly reduced food intake for the first 2 h following a fast in control mice, but this effect was no longer present by 4 hours. In *Magel2*-null mice, MT-II reduced food intake to a greater extent and this effect was still evident 24 hours after injection. Values are means ± SEM. *$p<0.05$, compared between genotypes by Student's t-test.

4.4 Discussion

Mice lacking *Magel2* have increased adiposity with proportionately increased leptin, suggesting leptin insensitivity (Bischof *et al.*, 2007, Kozlov *et al.*, 2007). Here, we show that *Magel2*-null mice are physiologically resistant to the effects of exogenously applied leptin, both before and after the onset of increased adiposity. Further, this leptin resistance is accompanied by a 39% reduction in the number of POMC neurons in the ARC, and by a complete absence of leptin-induced depolarization responses in the remaining POMC neurons. *Magel2* is therefore essential for normal leptin signaling in POMC neurons, and for the differentiation, proliferation, or survival of this population of neurons. Loss of POMC neuronal activation is accompanied by an exaggerated anorexigenic response to exogenous melanocortins, suggesting compensatory upregulation of downstream melanocortin response pathways in *Magel2*-null mice. The role of *MAGEL2* in melanocortin-associated neuronal pathways may provide important insights into dysfunctional ingestive behaviour and obesity in Prader-Willi syndrome.

Insensitivity to peripheral leptin has been demonstrated in diet-induced and genetic models of obesity (El-Haschimi *et al.*, 2000, Rahmouni *et al.*, 2008, Prpic *et al.*, 2003, Lin *et al.*, 2000). In principal, a failure to respond to acutely or chronically elevated leptin could be caused by reduced transport across the blood brain barrier, or by an intrinsic defect in leptin-responsive neurons. In the latter case, leptin insensitivity could be caused by failure of leptin either to inhibit the orexigenic drive (in NPY neurons), or to activate the anorexigenic drive (through

POMC neurons), or both mechanisms, as in congenital leptin insensitivity in mice carrying an inactive form of the leptin receptor (*LepR*^{db} mice). Although the anorexic response to peripherally administered leptin is absent in the *Magel2*-null mice, the electrophysiology results demonstrate that many arcuate hypothalamic neurons that express the leptin receptor remain leptin sensitive. Specifically, *Magel2*-null ARC slices have a similar the number of neurons displaying inhibitory responses to leptin as slices from control animals, and these responses are of similar amplitude. Moreover, the remaining POMC neurons retain sensitivity to NPY, so the loss of the leptin-mediated excitatory response is not indicative of a global cellular defect within the ARC. This retention of leptin-mediated inhibitory responses is consistent with the modest level of obesity in *Magel2*-null mice compared with that seen in the total leptin impairment present in *Lep*^{ob} mice, which lack leptin, or *Lepr*^{db} mice.

Indeed, several mouse strains have been constructed in which leptin signaling pathways are selectively impaired in POMC neurons. Mice engineered without leptin receptor expression only in POMC neurons display a mild obese phenotype, with a large increase in fat mass (Balthasar *et al.*, 2004, van de Wall *et al.*, 2008), similar in magnitude to that previously reported in *Magel2*-null mice (Bischof *et al.*, 2007). A similar degree of obesity and adiposity is seen with the inactivation of STAT3 in POMC neurons (Xu *et al.*, 2007). Unlike the *Magel2*-null mice, the POMC-STAT3 mutants remain sensitive to peripheral leptin, however they display defects in compensatory refeeding following food deprivation leading to reduced weight regain, similar to what we have observed in

Magel2-null mice (Xu *et al.*, 2007). Rapid effects of leptin action on ARC leptin receptors have been linked to increased PI3K signaling (Morrison *et al.*, 2005)(Morrison *et al.*, 2005, Xu *et al.*, 2005b). Accordingly, pharmacological blockade of PI3K signaling inhibits leptin induced activation of POMC neurons (Hill *et al.*, 2008). Targeted deletion of PI3K signaling in POMC neurons also eliminates leptin induced activation of POMC neurons, and significantly blunts the reduction in food intake seen with intracerebroventricular leptin administration (Hill *et al.*, 2008). Interestingly, these mice do not appear to have any defects in weight gain or body composition, though a different strategy aimed at downregulation of PI3K in POMC neurons does lead to a modest obesity phenotype and increased sensitivity to DIO (Hill *et al.*, 2009). Compared with the present results shown in *Magel2*-null mice, investigating a role for *Magel2* in PI3K signaling is warranted.

Several possible underlying causes might explain the complete absence of depolarizing responses to leptin seen in the *Magel2*-null mice. One might be a reduction in the numbers of POMC neurons that express the leptin receptor itself. Approximately half the number of additional neurons expressed leptin-induced pSTAT3 in the ARC of *Magel2*-null mice. Furthermore, we observed fewer GFP-expressing neurons in the ARC of *Magel2*xPOMC^{EGFP} than in control POMC^{EGFP} mice wildtype for *Magel2*. While we also observed fewer cells expressing c-fos in ARC of *Magel2*-null mice than in controls, this was also true in animals not receiving leptin, and the net increase with leptin of c-fos-expressing neurons was not different. Nonetheless, the reductions in numbers do not account for the

complete absence of excitatory electrical responses to leptin observed in POMC neurons.

Excitatory responses to leptin are mediated by a complex signaling cascade in POMC neurons (Qiu *et al.*, 2010). Activation of LepRb results in sequential activation of Jak2, phosphatidyl inositol-3-kinase (PI3K) and phospholipase γ1 (PLCγ1), which in turn operates a nonselective cation channel of the TRPC family (Qiu *et al.*, 2010). Given the complexity of this intracellular signaling pathway, we speculate that that Magel2 may participate in this cascade. Consistent with this hypothesis, preliminary investigations of leptin-activated neurons in the ventromedial nucleus (VMN) also points to a specific defect in depolarization responses. In the VMN, some neurons depolarize in response to leptin, rare cells hyperpolarize, and the majority of cells do not respond to leptin administration (Chee *et al.*, 2010, Shiraishi *et al.*, 2000, Dhillon *et al.*, 2006). Although we found this to be the case in control VMN neurons, VMN neurons from *Magel2*-null mice appear deficient in depolarizing responses suggesting that Magel2 is likely to be important for the integrity of the LepRb-TRPC signaling cascade in several neurochemical subtypes of neurons.

Fasting in rodents induces a state of negative energy balance that is reflected by dramatic decreases in circulating leptin levels (Ahima and Lazar, 2008, Frederich *et al.*, 1995, Ahima *et al.*, 1996) and compensatory hyperphagia on re-feeding. Deficiencies in fasting-induced hyperphagia and compensatory weight gain are found in models of POMC neuronal degradation or in POMC-specific STAT3 mutant mice (Xu *et al.*, 2005a, Xu *et al.*, 2007). Thus, appropriate

regulation of POMC neurons in the ARC is critical to normal responses to food deprivation, which are clearly impaired in *Magel2*-null mice. Other hypothalamic pathways could also contribute to dysfunctional feeding behaviour in *Magel2*-null mice. For example, orexin neurons normally activate NPY and inhibit POMC neurons to stimulate increases in food intake (Muroya *et al.*, 2004), and ablation of orexin neurons in the lateral hypothalamus causes a loss of fasting-induced arousal and defense of body weight during fasting (Yamanaka *et al.*, 2003). In fact, *Magel2*-null mice have fewer orexin neurons and a reduction in hypothalamic levels of orexin-A neuropeptide (Kozlov *et al.*, 2007, Schaller *et al.*, 2010), which could contribute to the impaired compensatory hyperphagia responses in the *Magel2* null mice.

Notably, the anorexic response to melanocortins is intact and indeed hyperactivated in *Magel2*-null mice, suggesting that melanocortin receptors in the paraventricular nucleus are not impaired by loss of Magel2. Further examination of melanocortin responsiveness in *Magel2*-null mice could provide compelling evidence for potential therapeutic intervention in PWS. The exact biochemical roles of Magel2 and how it participates in neuronal differentiation and/or survival as well as cellular activation in response to leptin remain to be determined. In summary, our results demonstrate that expression of *Magel2* is critical for leptin responses in POMC neurons in the ARC and for energy homeostasis in mice. It will be important to address whether loss of MAGEL2 in people with PWS likewise contributes to disrupted ingestive behaviour and energy homeostasis in this disorder.

4.5 Bibliography

Acuna-Goycolea,C. and van den Pol,A. N. (2005) "Peptide YY(3-36) inhibits both anorexigenic proopiomelanocortin and orexigenic neuropeptide Y neurons: implications for hypothalamic regulation of energy homeostasis." J.Neurosci. **25**(45):10510-10519.

Ahima,R. S. and Lazar,M. A. (2008) "Adipokines and the peripheral and neural control of energy balance." Mol.Endocrinol. **22**(5):1023-1031.

Ahima,R. S., Prabakaran,D., Mantzoros,C., *et al.* (1996) "Role of leptin in the neuroendocrine response to fasting." Nature **382**(6588):250-252.

Balthasar,N., Coppari,R., McMinn,J., *et al.* (2004) "Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis." Neuron **42**(6):983-991.

Bannon,A. W., Seda,J., Carmouche,M., *et al.* (2000) "Behavioral characterization of neuropeptide Y knockout mice." Brain Res. **868**(1):79-87.

Bischof,J. M., Stewart,C. L. and Wevrick,R. (2007) "Inactivation of the mouse Magel2 gene results in growth abnormalities similar to Prader-Willi syndrome." Hum.Mol.Genet. **16**(22):2713-2719.

Bjorbaek,C., Uotani,S., da Silva,B. and Flier,J. S. (1997) "Divergent signaling capacities of the long and short isoforms of the leptin receptor." J.Biol.Chem. **272**(51):32686-32695.

Cassidy,S. B. and Driscoll,D. J. (2009) "Prader-Willi syndrome." Eur.J.Hum.Genet. **17**(1):3-13.

Cassidy,S. B. (1997) "Prader-Willi syndrome." J.Med.Genet. **34**(11):917-923.

Challis,B. G., Coll,A. P., Yeo,G. S., *et al.* (2004) "Mice lacking pro-opiomelanocortin are sensitive to high-fat feeding but respond normally to the acute anorectic effects of peptide-YY(3-36)." Proc.Natl.Acad.Sci.U.S.A. **101**(13):4695-4700.

Chee,M. J., Price,C. J., Statnick,M. A. and Colmers,W. F. (2011) "Nociceptin/orphanin FQ suppresses the excitability of neurons in the ventromedial nucleus of the hypothalamus." J.Physiol. **589**(Pt 13):3103-3114.

Chee,M. J., Myers,M. G.,Jr, Price,C. J. and Colmers,W. F. (2010) "Neuropeptide Y suppresses anorexigenic output from the ventromedial nucleus of the hypothalamus." J.Neurosci. **30**(9):3380-3390.

Clement,K., Vaisse,C., Lahlou,N., *et al.* (1998) "A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction." Nature **392**(6674):398-401.

Coll,A. P., Challis,B. G., Lopez,M., Piper,S., Yeo,G. S. and O'Rahilly,S. (2005) "Proopiomelanocortin-deficient mice are hypersensitive to the adverse metabolic effects of glucocorticoids." Diabetes **54**(8):2269-2276.

Cowley,M. A., Smart,J. L., Rubinstein,M., *et al.* (2001) "Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus." Nature **411**(6836):480-484.

Cowley,M. A., Pronchuk,N., Fan,W., Dinulescu,D. M., Colmers,W. F. and Cone,R. D. (1999) "Integration of NPY, AGRP, and melanocortin signals in the hypothalamic paraventricular nucleus: evidence of a cellular basis for the adipostat." Neuron **24**(1):155-163.

Creemers,J. W., Lee,Y. S., Oliver,R. L., *et al.* (2008) "Mutations in the amino-terminal region of proopiomelanocortin (POMC) in patients with early-onset obesity impair POMC sorting to the regulated secretory pathway." J.Clin.Endocrinol.Metab. **93**(11):4494-4499.

Dhillon,H., Zigman,J. M., Ye,C., *et al.* (2006) "Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis." Neuron **49**(2):191-203.

Dragunow,M. and Faull,R. (1989) "The use of c-fos as a metabolic marker in neuronal pathway tracing." J.Neurosci.Methods **29**(3):261-265.

El-Haschimi,K., Pierroz,D. D., Hileman,S. M., Bjorbaek,C. and Flier,J. S. (2000) "Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity." J.Clin.Invest. **105**(12):1827-1832.

Elias,C. F., Aschkenasi,C., Lee,C., *et al.* (1999) "Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area." Neuron **23**(4):775-786.

Enriori,P. J., Evans,A. E., Sinnayah,P., *et al.* (2007) "Diet-induced obesity causes severe but reversible leptin resistance in arcuate melanocortin neurons." Cell.Metab. **5**(3):181-194.

Farooqi,I. S., Drop,S., Clements,A., *et al.* (2006) "Heterozygosity for a POMC-null mutation and increased obesity risk in humans." Diabetes **55**(9):2549-2553.

Farooqi,I. S., Keogh,J. M., Yeo,G. S., Lank,E. J., Cheetham,T. and O'Rahilly,S. (2003) "Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene." N.Engl.J.Med. **348**(12):1085-1095.

Frederich,R. C., Lollmann,B., Hamann,A., *et al.* (1995) "Expression of ob mRNA and its encoded protein in rodents. Impact of nutrition and obesity." J.Clin.Invest. **96**(3):1658-1663.

Graham,M., Shutter,J. R., Sarmiento,U., Sarosi,I. and Stark,K. L. (1997) "Overexpression of Agrt leads to obesity in transgenic mice." Nat.Genet. **17**(3):273-274.

Gunay-Aygun,M., Schwartz,S., Heeger,S., O'Riordan,M. A. and Cassidy,S. B. (2001) "The changing purpose of Prader-Willi syndrome clinical diagnostic criteria and proposed revised criteria." Pediatrics **108**(5):E92.

Hansen,M. J., Ball,M. J. and Morris,M. J. (2001) "Enhanced inhibitory feeding response to alpha-melanocyte stimulating hormone in the diet-induced obese rat." Brain Res. **892**(1):130-137.

Hill,J. W., Xu,Y., Preitner,F., *et al.* (2009) "Phosphatidyl inositol 3-kinase signaling in hypothalamic proopiomelanocortin neurons contributes to the regulation of glucose homeostasis." Endocrinology **150**(11):4874-4882.

Hill,J. W., Williams,K. W., Ye,C., *et al.* (2008) "Acute effects of leptin require PI3K signaling in hypothalamic proopiomelanocortin neurons in mice." J.Clin.Invest. **118**(5):1796-1805.

Hruby,V. J., Lu,D., Sharma,S. D., *et al.* (1995) "Cyclic lactam alpha-melanotropin analogues of Ac-Nle4-cyclo[Asp5, D-Phe7,Lys10] alpha-melanocyte-stimulating hormone-(4-10)-NH2 with bulky aromatic amino acids at position 7 show high antagonist potency and selectivity at specific melanocortin receptors." J.Med.Chem. **38**(18):3454-3461.

Israel,D. and Chua,S.,Jr. (2010) "Leptin receptor modulation of adiposity and fertility." Trends Endocrinol.Metab. **21**(1):10-16.

Kozlov,S. V., Bogenpohl,J. W., Howell,M. P., *et al.* (2007) "The imprinted gene Magel2 regulates normal circadian output." Nat.Genet. **39**(10):1266-1272.

Krude,H., Biebermann,H., Schnabel,D., *et al.* (2003) "Obesity due to proopiomelanocortin deficiency: three new cases and treatment trials with thyroid hormone and ACTH4-10." J.Clin.Endocrinol.Metab. **88**(10):4633-4640.

Leshan,R. L., Louis,G. W., Jo,Y. H., Rhodes,C. J., Munzberg,H. and Myers,M. G.,Jr. (2009) "Direct innervation of GnRH neurons by metabolic- and sexual

odorant-sensing leptin receptor neurons in the hypothalamic ventral premammillary nucleus." J.Neurosci. **29**(10):3138-3147.

Leshan,R. L., Bjornholm,M., Munzberg,H. and Myers,M. G.,Jr. (2006) "Leptin receptor signaling and action in the central nervous system." Obesity (Silver Spring) **14 Suppl 5**:208S-212S.

Li,G., Zhang,Y., Wilsey,J. T. and Scarpace,P. J. (2004) "Unabated anorexic and enhanced thermogenic responses to melanotan II in diet-induced obese rats despite reduced melanocortin 3 and 4 receptor expression." J.Endocrinol. **182**(1):123-132.

Lin,S., Thomas,T. C., Storlien,L. H. and Huang,X. F. (2000) "Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice." Int.J.Obes.Relat.Metab.Disord. **24**(5):639-646.

Mercer,R. E. and Wevrick,R. (2009) "Loss of magel2, a candidate gene for features of prader-willi syndrome, impairs reproductive function in mice." PLoS ONE **4**(1):e4291.

Montague,C. T., Farooqi,I. S., Whitehead,J. P., *et al.* (1997) "Congenital leptin deficiency is associated with severe early-onset obesity in humans." Nature **387**(6636):903-908.

Morrison,C. D., Morton,G. J., Niswender,K. D., Gelling,R. W. and Schwartz,M. W. (2005) "Leptin inhibits hypothalamic Npy and Agrp gene expression via a mechanism that requires phosphatidylinositol 3-OH-kinase signaling." Am.J.Physiol.Endocrinol.Metab. **289**(6):E1051-7.

Munzberg,H., Flier,J. S. and Bjorbaek,C. (2004) "Region-specific leptin resistance within the hypothalamus of diet-induced obese mice." Endocrinology **145**(11):4880-4889.

Munzberg,H., Huo,L., Nillni,E. A., Hollenberg,A. N. and Bjorbaek,C. (2003) "Role of signal transducer and activator of transcription 3 in regulation of hypothalamic proopiomelanocortin gene expression by leptin." Endocrinology **144**(5):2121-2131.

Muroya,S., Funahashi,H., Yamanaka,A., *et al.* (2004) "Orexins (hypocretins) directly interact with neuropeptide Y, POMC and glucose-responsive neurons to regulate Ca 2+ signaling in a reciprocal manner to leptin: orexigenic neuronal pathways in the mediobasal hypothalamus." Eur.J.Neurosci. **19**(6):1524-1534.

Ollmann,M. M., Wilson,B. D., Yang,Y. K., *et al.* (1997) "Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein." Science **278**(5335):135-138.

Patel,H. R., Qi,Y., Hawkins,E. J., *et al.* (2006) "Neuropeptide Y deficiency attenuates responses to fasting and high-fat diet in obesity-prone mice." Diabetes **55**(11):3091-3098.

Piper,M. L., Unger,E. K., Myers,M. G.,Jr and Xu,A. W. (2008) "Specific physiological roles for signal transducer and activator of transcription 3 in leptin receptor-expressing neurons." Mol.Endocrinol. **22**(3):751-759.

Prpic,V., Watson,P. M., Frampton,I. C., Sabol,M. A., Jezek,G. E. and Gettys,T. W. (2003) "Differential mechanisms and development of leptin resistance in A/J versus C57BL/6J mice during diet-induced obesity." Endocrinology **144**(4):1155-1163.

Qiu,J., Fang,Y., Ronnekleiv,O. K. and Kelly,M. J. (2010) "Leptin excites proopiomelanocortin neurons via activation of TRPC channels." J.Neurosci. **30**(4):1560-1565.

Rahmouni,K., Fath,M. A., Seo,S., *et al.* (2008) "Leptin resistance contributes to obesity and hypertension in mouse models of Bardet-Biedl syndrome." J.Clin.Invest. **118**(4):1458-1467.

Roseberry,A. G., Liu,H., Jackson,A. C., Cai,X. and Friedman,J. M. (2004) "Neuropeptide Y-mediated inhibition of proopiomelanocortin neurons in the arcuate nucleus shows enhanced desensitization in ob/ob mice." Neuron **41**(5):711-722.

Rosen,G. D., Williams,A. G., Capra,J. A., *et al.* (2000) "The Mouse Brain Library @ www.mbl.org." Int Mouse Genome Conference 14: 166 **2012**(January)

Scarpace,P. J., Matheny,M., Zolotukhin,S., Tumer,N. and Zhang,Y. (2003) "Leptin-induced leptin resistant rats exhibit enhanced responses to the melanocortin agonist MT II." Neuropharmacology **45**(2):211-219.

Schaller,F., Watrin,F., Sturny,R., Massacrier,A., Szepetowski,P. and Muscatelli,F. (2010) "A single postnatal injection of oxytocin rescues the lethal feeding behaviour in mouse newborns deficient for the imprinted Magel2 gene." Hum.Mol.Genet. **19**(24):4895-4905.

Segal-Lieberman,G., Trombly,D. J., Juthani,V., Wang,X. and Maratos-Flier,E. (2003) "NPY ablation in C57BL/6 mice leads to mild obesity and to an impaired refeeding response to fasting." Am.J.Physiol.Endocrinol.Metab. **284**(6):E1131-9.

Seo,S., Guo,D. F., Bugge,K., Morgan,D. A., Rahmouni,K. and Sheffield,V. C. (2009) "Requirement of Bardet-Biedl syndrome proteins for leptin receptor signaling." Hum.Mol.Genet. **18**(7):1323-1331.

Shiraishi,T., Oomura,Y., Sasaki,K. and Wayner,M. J. (2000) "Effects of leptin and orexin-A on food intake and feeding related hypothalamic neurons." Physiol.Behav. **71**(3-4):251-261.

Smart,J. L., Tolle,V. and Low,M. J. (2006) "Glucocorticoids exacerbate obesity and insulin resistance in neuron-specific proopiomelanocortin-deficient mice." J.Clin.Invest. **116**(2):495-505.

Tiesjema,B., la Fleur,S. E., Luijendijk,M. C. and Adan,R. A. (2009) "Sustained NPY overexpression in the PVN results in obesity via temporarily increasing food intake." Obesity (Silver Spring) **17**(7):1448-1450.

van de Wall,E., Leshan,R., Xu,A. W., *et al.* (2008) "Collective and individual functions of leptin receptor modulated neurons controlling metabolism and ingestion." Endocrinology **149**(4):1773-1785.

van den Top,M., Lee,K., Whyment,A. D., Blanks,A. M. and Spanswick,D. (2004) "Orexigen-sensitive NPY/AgRP pacemaker neurons in the hypothalamic arcuate nucleus." Nat.Neurosci. **7**(5):493-494.

Van Heek,M., Compton,D. S., France,C. F., *et al.* (1997) "Diet-induced obese mice develop peripheral, but not central, resistance to leptin." J.Clin.Invest. **99**(3):385-390.

Williams,K. W., Margatho,L. O., Lee,C. E., *et al.* (2010) "Segregation of acute leptin and insulin effects in distinct populations of arcuate proopiomelanocortin neurons." J.Neurosci. **30**(7):2472-2479.

Xu,A. W., Ste-Marie,L., Kaelin,C. B. and Barsh,G. S. (2007) "Inactivation of signal transducer and activator of transcription 3 in proopiomelanocortin (Pomc) neurons causes decreased pomc expression, mild obesity, and defects in compensatory refeeding." Endocrinology **148**(1):72-80.

Xu,A. W., Kaelin,C. B., Morton,G. J., *et al.* (2005a) "Effects of hypothalamic neurodegeneration on energy balance." PLoS Biol. **3**(12):e415.

Xu,A. W., Kaelin,C. B., Takeda,K., Akira,S., Schwartz,M. W. and Barsh,G. S. (2005b) "PI3K integrates the action of insulin and leptin on hypothalamic neurons." J.Clin.Invest. **115**(4):951-958.

Yamanaka,A., Beuckmann,C. T., Willie,J. T., *et al.* (2003) "Hypothalamic orexin neurons regulate arousal according to energy balance in mice." Neuron **38**(5):701-713.

Yaswen,L., Diehl,N., Brennan,M. B. and Hochgeschwender,U. (1999) "Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin." Nat.Med. **5**(9):1066-1070.

Chapter 5: Conclusion

When I joined the Wevrick laboratory, the *Magel2*-null mouse was newly developed, and no published data existed on its neonatal or adult phenotypes. Two projects were underway, one in the laboratory of our collaborator Dr. Colin Stewart, examining activity rhythms in *Magel2*-null mice based on the circadian expression of high *Magel2* levels in the SCN, and the second in the Wevrick laboratory examining growth and metabolism in the *Magel2*-null mice. Both of these studies were fruitful, and were published in 2007 (Kozlov *et al.*, 2007, Bischof *et al.*, 2007).

My first experimental examination of the *Magel2*-null mice involved the daily weighing of neonatal pups, and the weekly weighing of adult mice to help determine growth differences between *Magel2*-null and control mice. Indeed, we were able to demonstrate that similar to PWS, *Magel2*-null mice were underweight prior to weaning, and overweight as adults (Bischof *et al.*, 2007). The adult *Magel2*-null phenotype was coupled with a notable change in body composition, with significantly increased adipose tissue mass and reduced lean mass, which is also reminiscent of PWS (Butler *et al.*, 2007, Reus *et al.*, 2011). This type of body composition defect suggests reduced resting metabolic rate, and in support of this idea, it was also shown that the *Magel2*-null mice gain their excess weight despite consuming less food than control mice (Bischof *et al.*, 2007). The *Magel2*-null mice are not truly hypophagic however, as their reduction in food intake (~10%) is not great enough to compensate for the large reductions in their voluntary activity levels (Kozlov *et al.*, 2007). Taken together, it is clear

that the obesity seen in *Magel2*-null mice is due to overall reductions in energy expenditure.

This early study completed in the Wevrick Laboratory led to my further investigations on *Magel2*-null mice which have been described in this thesis. Differences in responsiveness during handling and weighing of the mice initiated the behavioural and neurochemical studies described in Chapter 2; difficulties in breeding from *Magel2*-null males compared to carrier males to obtain pups for weighing initiated the reproductive studies described in Chapter 3; and curiosity about the mechanisms of weight gain and adiposity led to the leptin-focused studies described in Chapter 4. I believe that these studies highlight that the *Magel2*-null mouse is indeed a valuable and appropriate model for numerous PWS phenotypes, and thus, that *MAGEL2* plays a notable role in the etiology of PWS. Overall results and insights from these studies are highlighted below.

5.1 Loss of *Magel2* Causes Brain Volume, Neurochemical, and Behavioural Changes

5.1.1 Overall and regional reductions in brain volume in Magel2-null mice

Using high resolution MRI, our collaborators found that the total brain volume in *Magel2*-null mice was reduced by approximately 3-4% compared to control mice. Specifically, reductions of ~4-5% in volume were observed in the parieto-temporal lobe, amygdala, dentate gyrus, nucleus accumbens, olfactory bulb, and corpus callosum (Figure 2.2). These brain areas are distinct from the brain regions that have been identified as abnormal or reduced in PWS (Hashimoto *et al.*, 1998, Miller *et al.*, 2007a, Miller *et al.*, 2007b). Notably, no

structural differences were observed in the hypothalamus or pituitary glands of *Magel2*-null mice. It is likely that the specific changes seen in the brains of people with PWS are caused or contributed to by the loss of other PWS candidate genes, for example, enlarged ventricles have been observed in *Ndn*-null mice (Lee *et al.*, 2005).

Despite their dissimilarity with regional reductions in brain volume seen in PWS, the regional reductions seen in *Magel2*-null mice have important implications for brain development and function in these animals. Two of the regions of reduced brain volume seen in *Magel2*-null mice, the amygdala and the nucleus accumbens, are known to play key roles in the development of anxiety. Interestingly, anxiety in people with panic disorder have been associated with reductions in amygdala volume (Hayano *et al.*, 2009). It is possible that reductions in these brain areas may contribute to the anxious phenotype observed in *Magel2*-null mice. Despite these possibilities, the contribution of regionally reduced brain volume to physiological phenotypes observed in *Magel2*-null mice is unclear.

5.1.2 Altered serotonin and dopamine levels in Magel2-null mice

To examine biogenic amines and their metabolites, as well as specific amino acid levels in the brains of both late-embryo and adult *Magel2*-null mice, brains were sub-dissected into various regions, and processed through high performance liquid chromatography with fluorescence detection. No differences were detected between *Magel2*-null and control embryos at E18 (Table 2.2), though only cortex and cerebellum were examined. In contrast, in *Magel2*-null

male mice aged 20-24 weeks, there were reductions in both dopamine (DA), serotonin (5HT), and the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) seen in discrete brain regions. DA was reduced by approximately 40% in the hypothalamus, where 5HT was reduced by 16, 25, and 30% in the prefrontal cortex, cortex, and hypothalamus, respectively. 5-HIAA levels were comparable between *Magel2*-null and control in the prefrontal cortex, but reduced 24 and 19% in the cortex and hypothalamus (Table 2.2). Despite the reductions in DA and 5HT observed, Magel2-null mice had comparable numbers of DA and 5HT producing neurons in the hypothalamus and brainstem (Figure 2.3), suggesting the neurochemical reductions are due to a functional vs. a structural defect. This finding has intriguing implications towards specific phenotypes seen in the *Magel2*-null mouse, and perhaps people with PWS.

Most of the DA present in the hypothalamus is produced locally, in specialized neurons with cell bodies mostly found in the ARC (Bjorklund and Dunnett, 2007, van den Pol *et al.*, 1984). Dendrites of these neurons project throughout the hypothalamus, and major axons extend into the ME to release DA into the hypophyseal portal blood (van den Pol *et al.*, 1984, Ershov *et al.*, 2002). Here, the major effect of DA is to inhibit the release of prolactin (Lamberts and Macleod, 1990), but it has also been shown to stimulate GH secretion through regulation of GHRH neurons (Garcia-Tornadu *et al.*, 2010). Elevated levels of circulating prolactin impair the HPG axis via GnRH neurons, and lead to irregular cycling and anovulation in females and hypogonadism in men (Bolyakov and Paduch, 2011, Bachelot and Binart, 2007). Therefore, reduced hypothalamic

dopamine in the *Magel2*-null mouse may contribute to their fertility defects (see Chapter 3), and perhaps the reduced GHRH responsiveness that has been described in these mice as well (Tennese and Wevrick, 2011).

Within the hypothalamus, DA neurons send projections to the VMH, LH, DMH, PVN, and elsewhere (van den Pol *et al.*, 1984). In both the VMH and LH, DA release has been linked to the regulation of meal size and number (Fetissov *et al.*, 2002, Meguid *et al.*, 1995, Meguid *et al.*, 1997), suggesting a potential role for reduced hypothalamic DA in the food intake alterations observed in *Magel2*-null mice (Kozlov *et al.*, 2007, Bischof *et al.*, 2007).

In the brain, 5HT plays a critically important function in the regulation of numerous functions, including regulating mood and behaviour, reproduction, and food intake and body weight. 5HT regulates these actions by acting through a number of different receptor types. The 5HT 2C receptor ($5HT_{2C}R$) is highly expressed in the hypothalamus, and has clear roles in regulating energy homeostasis within the ARC and PVN (Heisler *et al.*, 2003, Heisler *et al.*, 2006, Lam *et al.*, 2008), and in the regulation of the hypothalamic-pituitary-adrenal axis (Heisler *et al.*, 2007). Notably, $5HT_{2C}R$ is differentially edited, and the isoforms present in people with PWS differ from those found in control individuals (Kishore and Stamm, 2006). Changes in $5HT_{2C}R$ editing have been found in transgenic mice with a PWS IC deletion, which leads to loss of expression of genes in the PWS region (Kishore *et al.*, 2010, Doe *et al.*, 2009). Mice with altered $5HT_{2C}R$ RNA editing display phenotypes reminiscent of PWS including neonatal hypotonia, growth deficiency, obesity, and hyperphagia (Doe *et al.*,

2009, Morabito *et al.*, 2010). The reduction of 5HT release seen in *Magel2*-null mice coupled with abnormal $5HT_{2C}R$ editing seen in PWS IC deletion mice may have strong implications in the etiology of PWS. In support of this, treatment of people with PWS with selective serotonin reuptake inhibitors (SSRIs), has been associated with a decrease in skin-picking and OCD-like behaviour (Hellings and Warnock, 1994, Warnock and Kestenbaum, 1992), and the onset of menses (Warnock *et al.*, 1995), though these findings are not consistent among all people with PWS. Still, it is clear that the 5HT system plays a role in PWS, and appears to be at least partially regulated by *Magel2*.

5.1.3 Abnormal behaviour in Magel2-null mice

We subjected the *Magel2*-null mice to a number of behavioural tests, performed both in house and at a specialized centre for rodent behavioural studies. Though reductions in locomotor behaviour were confirmed (Figure 2.4A-C), we did not observe any significant changes consistent with reductions in muscle function (Figure 2.4D-E), learning, or memory in the *Magel2*-null mice (Figure 2.5A). However, we did observe anxiety-like behaviour in *Magel2*-null females, with reduced marble burying and avoidance of novel objects (Figure 2.5B-D). Interestingly, sex-specific differences in anxiety and novelty behaviour have been observed in a model of serotonin depletion (Hohmann *et al.*, 2007), but in this case, the male mice were affected while female animals did not display a behavioural change. We were unable to detect any evidence of obsessive or compulsive behaviour in the *Magel2*-null mice, though the behavioural testing undertaken did not specifically target this type of behaviour. Additional

behavioural tests such as home cage grooming activity over time, and closer analysis of locomotion patterns could clarify the presence or absence of obsessive/compulsive behaviour in *Magel2*-null mice (Wang *et al.*, 2009).

5.2 Loss of *Magel2* Causes Reproductive Impairment

5.2.1 Puberty is delayed and elongated in Magel2-null females

There is some controversy about the observations used to examine puberty in laboratory mice, but it is generally accepted that the age at vaginal opening is a good indicator of pubertal initiation, and the age of vaginal estrus are useful surrogate markers in female animals (Safranski *et al.*, 1993). Evaluation in males is more complicated with less obvious external signs of puberty, though age of preputial separation is occasionally used as a surrogate marker (Korenbrot *et al.*, 1977), despite being poorly coordinated with elevating testosterone levels and sexual behaviour. As a result, I limited my pubertal examinations in *Magel2*-null mice to females, and found a delay in the timing of vaginal opening, and an extended time period between vaginal opening and the appearance of vaginal estrus (Table 3.1), suggesting impaired regulation of puberty in mice missing *Magel2*. Changes in pubertal onset and duration have not been identified in other circadian rhythm mutant mice (Miller *et al.*, 2004), suggesting that this phenotype in the *Magel2*-null mice is not simply a consequence of disrupted circadian rhythm.

Onset of puberty has long been linked to appropriate stores of energy in both rodents and humans (Kennedy., 1969, Frisch and McArthur, 1974), and it is clear that the adipose derived hormone leptin plays a central role, with mice

lacking either leptin or its receptor being infertile and failing to initiate puberty, and leptin treatment rapidly inducing puberty in adult mice lacking leptin (Chehab *et al.*, 1996). Low doses of leptin have also been shown to induce early puberty in normal mice (Ahima *et al.*, 1997, Chehab *et al.*, 1997). These findings support the hypothesis that *Magel2*-null mice may have impairments in leptin signaling pathways that contribute to their reproductive problems, including altered initiation of puberty and early onset infertility.

5.2.2 Magel2-null mice show early reproductive decline and infertility by 24 weeks of age

To examine the effect of *Magel2* on fertility, I paired *Magel2*-null mice of both genders with young, control C57BL/6 mates with proven fertility, and measured the proportion of pairs who successfully produced litters, the length of time between pairing and mating, and the size and survival of litters born. Normal C57BL/6 mice have a high fertility rate and are identified by the Jackson Laboratory (stock #000664) as excellent breeders, reliably producing litters up to and beyond 7 months of age. In line with this, I observed fertility rates among control littermates to be greater than 80% between the ages of 7 and 35 weeks (Figure 3.1A). In contrast, at even young ages (7-14 weeks), the proportion of *Magel2*-null sires or dams producing litters was decreased compared to control pairings, and the fertility rate of both *Magel2*-null males and females showed significant decline by 19-24 weeks of age, with fertility rates lower than 25%. Strikingly, neither male nor female *Magel2*-null mice were able to produce litters beyond 24 weeks of age, indicating infertility (Figure 3.1A).

C57BL/6 mice show an age-related decline in fertility beginning at about 6 months of age. This decline is typically associated with gonadal changes including a reduction in developing follicles in females (Gosden *et al.*, 1983), and increased Leydig cell number and seminiferous tubule vacuolization in males (Takano and Abe, 1987). Similar changes were not seen in the *Magel2*-null mice (Figures 3.2, 3.3), indicating that the reproductive decline and infertility seen in these animals is not a result of premature aging of the reproductive system. Female *Magel2*-null mice show normal numbers and development of ovarian follicles, but do show a notable absence of corpora lutea in their ovaries by 24 weeks of age (Figure 3.3D), suggesting that a loss of ovulation likely underlies their infertility. No histological abnormalities are seen in the testes of *Magel2*-null mice, and they appear to produce normal numbers of healthy motile sperm, suggesting that their infertility is not due to gonadal changes. Taken together, these results indicate that infertility in these mice is related to central components of the HPG axis.

Among the *Magel2*-null mice that were fertile, I also noticed significant lengthening of the pairing to mating interval in both males and females. In the female *Magel2*-null mice this can be explained by their lengthened and irregular estrous cycles, which were frequently missing the proestrus stage (Figure 3.1B). The proestrus stage of the estrous cycle is the ovulatory stage, the absence of this stage indicating an absence of ovulation (Goldman *et al.*, 2007). In confirmation of this idea, I noted a number of ovaries in young *Magel2*-null females did not have corpora lutea, and a reduction in corpora lutea in the remaining ovaries

(Figure 3.3B), suggesting reduced ovulations. Since ovulation is triggered by a surge of LH acting on mature follicles, which are present in the *Magel2*-null females, it appears that LH surge generation is impaired in these mice, indicating reduced responsiveness of GnRH neurons to follicle derived estrogens, or a pituitary defect in the responsiveness to GnRH. The normal development of follicles, which occurs in response to FSH, in the *Magel2*-null mice, suggests that this defect is more likely related to impaired regulation of GnRH neurons in response to ovarian hormones. Impaired regulation of GnRH neurons in response to gonadal sex hormones is also suggested in the *Magel2*-null male mice which show reduced testosterone levels, despite normal levels of LH and FSH. If testosterone was properly feeding back to the hypothalamus, low testosterone levels would cause increased GnRH pulsatility that would then lead to elevated levels of LH and FSH. Taken together, these results suggest that impaired responsiveness of central components of the HPG axis are responsible for the reduced fertility rate and increased time between pairing and mating in *Magel2*-null mice.

Another factor contributing to the poor reproductive success in mice missing *Magel2* is likely the observed olfactory abnormality that develops after 10 weeks of age in both male and female mice (Figure 3.5), with increased latency to find a buried food item following a fast. Sexual motivation and successful reproduction in rodents is dependent on intact olfactory ability with anosmic mice failing to breed (Yoon *et al.*, 2005). This defect appears to have a motivational component, as *Magel2*-null mice are able to find an invisible vanilla-scented spot

placed in their cage, but spend much less time investigating this odor. Also, during olfactory preference testing, *Magel2*-null male mice clearly spend more time with soiled bedding, however, importantly, they show no preference for female estrus soiled bedding compared to male soiled bedding. A lack of female olfactory preference in males has been shown in non-copulating rats (Portillo and Paredes, 2004), and impaired olfaction in females can lead to dysregulated estrous cycling and poor maternal behaviour (Vandenbergh., 1973), which was also observed in *Magel2*-null females, who display frequent cannibalizations and dramatically reduced litter size at weaning compared to birth. Pheromone detection in the mouse has been shown to be a result of a direct connection between the main olfactory epithelium and GnRH neurons (Yoon *et al.*, 2005, Mandiyan *et al.*, 2005, Wang *et al.*, 2007), again suggesting improper regulation of GnRH neurons as a major contributor to the reproductive deficits seen in *Magel2*-null mice. Coupling reduced responsiveness of GnRH neurons resulting from Magel2 loss with the reduction in GnRH neuron number, and ME projections resulting from loss of *Ndn (Miller et al., 2008),* suggests that MAGEL2 and NDN may have an additive role in the completely infertile PWS phenotype.

5.3 Loss of *Magel2* Impairs Leptin Signaling

The regulation of energy balance by the hypothalamus is due to the coordinated action of specialized neurons which sense circulating signals of energy stores such as adipose derived hormone leptin (Ahima and Lazar, 2008). Leptin receptors (LepR) are located throughout the brain, and are present in at

least six different isoforms (LepRa-LepRf) (Fei *et al.*, 1997, Lee *et al.*, 1996). LepRb, also known as the long receptor isoform, is the signaling form of the LepR (Lee *et al.*, 1996, Baumann *et al.*, 1996), and is expressed at high levels in the hypothalamus, especially within the ARC, DMH, VMH, and MN (Elmquist *et al.*, 1998, Mercer *et al.*, 1996). Activation of LepRb by the binding of leptin activates numerous signaling pathways (Figure 5.1). The involvement of leptin in the regulation of energy balance and fertility, both of which are perturbed in *Magel2*-null mice (Bischof *et al.*, 2007)(Chapter 3 of this Thesis), and the overlap of LepRb expression with areas of high *Magel2* expression prompted an in depth study of leptin function in *Magel2*-null mice, focusing on the ARC, where leptin activates POMC neurons, and inhibits NPY neurons.

5.2.1 Magel2-null Mice are Resistant to Peripherally Administered Leptin

Magel2-null mice are overweight, with excessive adipose tissue as adults (Bischof *et al.*, 2007). I administered a 2.5 mg/kg dose of leptin to 20-24 week old control and *Magel2*-null mice, to see if I could observe reductions in food intake. While a significant reduction in food intake was seen in the control mice, no reduction was seen in *Magel2*-null mice (Figure 4.2B). This suggests leptin insensitivity, which was expected in these mice as at this age they are heavier than controls (Figure 4.2A) and (Bischof *et al.*, 2007), and leptin insensitivity has repeatedly been shown in both diet-induced and genetic models of obesity (El-Haschimi *et al.*, 2000, Prpic *et al.*, 2003, Lin *et al.*, 2000, Rahmouni *et al.*, 2008, Marsh *et al.*, 1999). What was unexpected, was the finding that *Magel2*-null mice are already insensitive to the peripheral anorexic effects of leptin at 6 weeks of

age (Figure 4.2D), when they are not different in weight from control mice. This suggests that the leptin insensitivity seen in *Magel2*-null mice is intrinsic, and directly due to the loss of *Magel2*. However, because the obesity seen in *Magel2*-null mice is moderate compared to the massive obesity seen in mice with complete leptin insensitivity due either to the loss of leptin itself (ob/ob mice (Ingalls *et al.*, 1950)) or the leptin receptor (db/db mice (Hummel *et al.*, 1966)) (Coleman., 2010), leptin appears to have some action in *Magel2*-null mice, despite no longer causing an acute reduction in food intake.

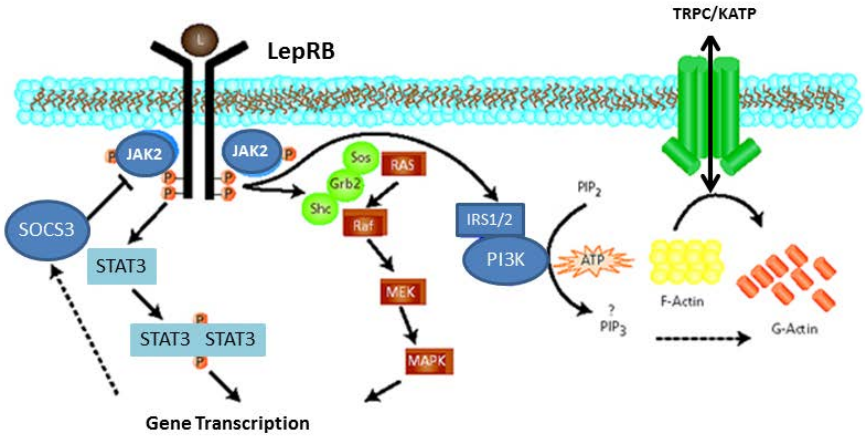


Figure 5.1 Leptin Signaling Pathways

Binding of leptin (L) to LepRb leads to phosphorylation (P) of the intracellular portion of the receptor, which stimulates numerous signaling pathways including JAK-STAT, MAPK, and PI3K. Activation of these pathways leads to changes in gene transcription and ion channel function. Increased SOCS3 production has been indicated as a negative regulator of continued JAK-STAT signaling.

Figure modified from Sigma-Aldrich "Effects of Peptides" website available at (http://www.sigmaaldrich.com/life-science/cell-biology/obesity-research/learning-center/effects-of-peptides.html)(2012); Adapted from (Harvey and Ashford, 2003).

To examine the ability of peripheral leptin to activate LepRb in the ARC, I measured levels of STAT3 phosphorylation (pSTAT3) with and without leptin treatment, and compared levels between control and *Magel2*-null mice. There was no difference in baseline pSTAT3 between *Magel2*-null and control mice, and though leptin caused a significant increase in pSTAT3 levels in both genotypes, levels in *Magel2*-null mice were lower than those seen in control mice (Figure 4.3). This supports the idea that *Magel2*-null mice maintain some response to peripheral leptin administration, but that they demonstrate a degree of leptin insensitivity due to *Magel2* loss.

5.2.2 Leptin fails to activate ARC POMC neurons in Magel2-null mice

To directly examine the responsiveness of LepRb+ neurons in the ARC, I bred *Magel2 +/-* carrier males with females homozygous for GFP expression in LepRb neurons ($LepRb^{EGFP}$) provided by Dr. Martin J. Myers (Leshan *et al.*, 2006). This cross produced both *Magel2*-null and control littermates, all of which express GFP in LepRb neurons. LepRb+ neurons in the ARC include NPY and POMC neurons, both of which are inhibited by NPY itself (Acuna-Goycolea and van den Pol, 2005, Ghamari-Langroudi *et al.*, 2005, Roseberry *et al.*, 2004). Using whole-cell visualized-patch recordings of GFP+ neurons, I determined that there is no difference in resting membrane potential (RMP) of *Magel2*-null and control $LepRb^{EGFP}$ mice (Figure 4.6C). There was also no difference in the magnitude of hyperpolarization induced by NPY in the GFP+ neurons (Figure 4.6E-F), providing evidence that ARC LepRb+ neurons are similar between *Magel2*-null and control mice, and that *Magel2* is not required for normal NPY

signaling. Since leptin normally depolarizes POMC neurons and hyperpolarizes NPY neurons (Cowley *et al.*, 2001, van den Top *et al.*, 2004), I expected a mixed population of responses in $LepRb^{EGFP}$ neurons. Indeed, this was the case in control mice, however the *Magel2*-null mice never exhibited depolarizing responses to leptin, suggesting a specific insensitivity to leptin in ARC neurons normally activated by leptin which include POMC neurons. This finding is in line with the lack of anorexia seen with peripherally administered leptin in the *Magel2*-null mice, as activation of POMC neurons is necessary for the release of the anorexigenic peptide α-MSH.

To confirm a defect in ARC POMC neurons in *Magel2*-null mice, I bred carrier *Magel2* +/- males with females that express GFP in POMC neurons ($POMC^{EGFP}$) (Cowley *et al.*, 2001). Again, no difference in RMP was observed in GFP+ neurons between *Magel2*-null and control mice, and these neurons responded normally to NPY (Figure 4.6J). As implicated in the $LepRb^{EGFP}$ mice, $POMC^{EGFP}$ neurons in *Magel2*-null mice were never activated by leptin (Figure 4.6K) reaffirming the specific role of *Magel2* in leptin signaling in POMC neurons. Further, I observed a reduction in the number of GFP+ neurons in *Magel2*-null mice, indicative of a loss of POMC neurons, suggesting that Magel2 or the leptin response it mediates may be important for the development or survival of this neuronal population in general. Further study is needed to determine when POMC neuron loss occurs, but since my experiments were completed in young (6-10 week) mice, the loss does not appear to be age-related or secondary to the development of obesity.

Other ARC neurons normally activated by leptin include kisspeptin neurons, which directly activate GnRH neurons to control puberty and fertility (Han *et al.*, 2005, Irwig *et al.*, 2004, Messager *et al.*, 2005). Since no activating leptin responses were seen in LepRb+ neurons in *Magel2*-null mice at all, rather than being specific to POMC neurons directly, it appears that Magel2 is involved in the transduction of stimulatory leptin responses in general. Further study into other LepRb+ expressing neuron populations throughout the brain, including those in the VMN, DMN, MN regions of the hypothalamus will better describe the role of Magel2 in leptin signaling.

5.4 Addressing Phenotypic Consequences of *Magel2*-loss

5.4.1 Potential Interventions in Magel2-null Mice

Because of the varied phenotypes identified in *Magel2*-null mice, individual targeting of each affected system is likely to produce the greatest recovery of normal physiology in these animals. A number of interventions come to mind to address some of the physiologic deficits encountered in *Magel2*-null mice, and some of these are discussed below.

Growth hormone treatment

GH treatment has greatly improved the quality of life of many people with PWS, with improved body composition and increased linear growth, increased physical activity, as well as improvements in behaviour, psychosocial development, and preservation of cognitive function (Carrel *et al.*, 2010, de Lind van Wijngaarden *et al.*, 2009, Sanchez-Ortiga *et al.*, 2011, Whitman *et al.*, 2002, Siemensma *et al.*, 2012). *Magel2*-null mice have demonstrated body composition

changes, with reduced lean mass and excess adipose tissue (Bischof *et al.*, 2007). They have also been demonstrated to have reductions in insulin-like growth factor 1 (IGF-1) levels, and defective ghrelin-stimulated GH release, but normal GH release following GHRH administration (Tennese and Wevrick, 2011). This suggests an intact pituitary store of GH, with hypothalamic dysregulation of GHRH neurons in *Magel2*-null mice. Administration of GH or GHRH analogues to GHRH knockout mice have shown effective in normalizing growth and body composition, though effects on physical activity were not measured (Alba *et al.*, 2005, Alba *et al.*, 2006). Based on this model, it is likely to predict that body composition may normalize in *Magel2*-null mice by reduceing adipose tissue mass and increasing lean mass; however, the current availability of GH treatment in people with PWS would not make this an effective use of research dollars.

Oxytocin treatment

Reductions in the number of OT neurons and the level of circulating OT have been observed in people with PWS (Swaab *et al.*, 1995, Hoybye., 2004). Recently, it has been shown that OT therapy in PWS had beneficial effects such as increasing trust and reducing disruptive behaviours (Tauber *et al.*, 2011). Reductions in hypothalamic OT have recently been reported in *Magel2*-null mice, and a single OT injection is reported to have dramatic effects on their immediate feeding ability and survival (Schaller *et al.*, 2010). In rodents, OT has demonstrated to play an important role in sexual motivation and copulatory behaviour in both male and female rodents, with administration of OT antagonists reducing mating behaviour (Borrow and Cameron, 2012, Waldherr and Neumann,

2007). OT also has demonstrated roles in promoting maternal care (Bosch and Neumann, 2012), a type of behaviour likely reduced in *Magel2*-null mice evidence by their high rates of litter cannibalization and reduced survival of pups to weaning (Chapter 3). It would be interesting to see if OT therapy in *Magel2*-null mice had beneficial effects on the, mating, mothering, or other behavioural defects observed. Such effects could strengthen the justification for OT therapy in people with PWS with an aim to improve social function and reduce behavioural issues.

Melanocortin treatment

In Chapter 4 of this thesis, I have shown that *Magel2*-null mice have reduced activation of POMC neurons in response to leptin. This suggests that these mice have reduced release of α-MSH in regions of the hypothalamus receiving POMC neuronal projections. Further, I demonstrated that administration of a melanocortin agonist, MT-II, has increased effectiveness in reducing food intake in *Magel2*-null mice compared to controls, indicating an upregulation of the melanocortin system downstream of POMC neurons. Repeated administration of MT-II has been shown to cause weight reduction in obese mice and rats (Strader *et al.*, 2007, Pierroz *et al.*, 2002, Li *et al.*, 2004), and intermittent administration appears to remain effective in the promotion of weight loss, reducing sensitization to repeated drug exposure (Zhang *et al.*, 2010). I predict that repeated dosing of overweight *Magel2*-null mice with MT-II will lead to weight loss and perhaps improvement in body composition. Additionally, it would make sense that weaning *Magel2*-null mice onto a regime of MT-II would prevent

their development of obesity, which is likely contributed to by their perceived energy deficit related to the insensitivity of POMC neurons to leptin. If correct, these hypotheses have strong implications for the use of melanocortin therapy to assist in the management of obesity in people with PWS. A concern with this type of therapy in obese and non-obese humans is the associated increase in blood pressure and heart rate seen with melanocortin agonists (Greenfield., 2011). This concern may be minimized in the PWS population if similar to *Magel2*-null mice, they display increased anorectic sensitivity to melanocortin agonism. This could lead to a lower dose or reduced dosing schedule of the melanocortin agonist preserving effects on weight loss while minimizing cardiovascular side effects.

It seems clear that addressing various physiologic concerns in *Magel2*-null mice is complicated by the breadth of the identified phenotypes, suggesting combinational therapy may be needed, as has been observed in people with PWS. Finding a cellular link between the systems affected by *Magel2* loss may identify pharmacological targets that would allow targeting of multiple systems at once. One intriguing possibility for this would be the manipulation of TRPC channel activation.

5.4.2 TRPC Channel Activation as a Potential Mediator of Magel2-related Phenotypes

Canonical transient receptor potential (TRPC) channels are a family of 7 members widely expressed in most tissues, including high levels in the brain and hypothalamus (Riccio *et al.*, 2002). TRPC channels are nonselective, calcium-permeable cation channels, and their activation leads to calcium influx into the

cells expressing them (Abramowitz and Birnbaumer, 2009). Impaired TRPC channel activation has been identified in a number of disease states, and a large number of diseases and complex syndromes map to chromosomal loci that contain TRP channel genes (Abramowitz and Birnbaumer, 2009, Abramowitz and Birnbaumer, 2007).

In the ARC, both POMC and kisspeptin neurons mediate their leptin induced depolarizations via activation of TRPC channels (Qiu *et al.*, 2010, Qiu *et al.*, 2011). 5HT action on $5HT_{2C}$Rs in POMC neurons has also been linked to TRPC channel activation. Outside of the ARC, TRPC channels have shown to be important in transmitting light-activated signals to the SCN in the regulation of circadian rhythms (Warren *et al.*, 2006), and in transmitting the kisspeptin signal to GnRH neurons in the POA (Zhang *et al.*, 2008). Leptin responses in the premammillary region of the hypothalamus has also been associated with TRPC channel activation (Williams *et al.*, 2011). Taken together, activation of TRPC channels has been implicated in the proper functioning of all the major systems that appear impaired by loss of *Magel2*, including those involved in circadian rhythm, fertility, and energy balance regulation. A key experiment that would lend strong support for the role of impaired TRPC channel activation in *Magel2*-null mice would be examining activation of GnRH neurons by kisspeptin using a similar strategy as the leptin study in $POMC^{EGFP}$ mice, as $GnRH^{EGFP}$ mice currently exist and are available for study (Spergel *et al.*, 1999). Investigation into the association of Magel2 with TRPC channel components and/or signaling regulators may establish a cellular role for Magel2, which has remained elusive

thus far. Pharmacologic targeting of TRPC channels is in its infancy, and a lack of specific agonists for treatment is a current limitation on the potential therapeutics of TRPC channel modulation (Abramowitz and Birnbaumer, 2009). Further identifying diseases and syndromes with affected TRPC channel activation will be an important driver in the development of these compounds.

5.5 Hypothalamic dysfunction in other forms of genetic obesity and their relation to PWS/*Magel2*-null mice

A number of other obesity syndromes, notably Bardet-Biedl Syndrome (BBS), display hypothalamic dysfunction. BBS is a heterogeneous genetic disorder with mutations in fifteen genes being described (Guo and Rahmouni, 2011). Aside from obesity, other features of BBS include retinopathy, renal defects, polydactyly, learning disabilities, and hypogonadism. BBS is caused by abnormal ciliary function, and BBS proteins typically localize to the basal body and ciliary axoneme (Guo and Rahmouni, 2011, Zaghloul and Katsanis, 2009). Both necdin and Magel2 have been shown to interact with BBS4 and BBS8 (Lee *et al.*, 2005), suggesting the cause of hypothalamic dysfunction and obesity may be similar between PWS and BBS. In mice, *Bbs2*, *Bbs4*, and *Bbs6* have demonstrated critical roles in leptin receptor signaling, especially related to the leptin-melanocortin system (Seo *et al.*, 2009). *Bbs2*, *Bbs4*, and *Bbs6* single deletion mice are hyperphagic and obese, with reduced locomotor activity, elevated leptin levels and impaired leptin responsiveness, and reduced POMC levels, suggesting BBS mediates obesity through disruption of the leptin-melanocortin signaling pathway (Rahmouni *et al.*, 2008). These findings are

highly similar to findings in *Magel2*-null mice, including those described in detail in Chapter 4, suggesting overlap in the mechanism of obesity in these mouse models, and the idea that PWS and BBS candidate genes may function together in various cellular processes.

Most single gene causes of obesity in humans also have underlying hypothalamic dysfunction. People with mutations in the leptin gene are severely obese with hyperphagia, hypogonadotropic hypogonadism, and delayed puberty (Montague *et al.*, 1997, Rau *et al.*, 1999, Gibson *et al.*, 2004). Similarly, leptin receptor deficiency leads to severe obesity, but also disrupted behaviour including social dysfunction and impulsivity and stubbornness, as well as delayed puberty and hypogonadotropic hypogonadism (Clement *et al.*, 1998, Farooqi *et al.*, 2007). These phenotypes are reminiscent of the PWS and *Magel2*-null mouse phenotypes, supportive of a role for leptin signaling pathways in the hypothalamus in the development of PWS.

In mice, loss of the transcription factor single-minded homolog 1 (*Sim1*) leads to significant hypoplasia of the hypothalamus, including complete loss of the SON and PVN, and early postnatal lethality (Michaud *et al.*, 1998). Because of the lethality of *Sim1-/-* mice, studies have used *Sim1+/-* mice to examine the function of *Sim1* in hypothalamic development and function. *Sim1+/-* mice are obese, hyperphagic, and hyperleptinemic (Michaud *et al.*, 2001), and have reduced expression of CRH, AVP, TRF and almost complete loss of OT expression (Kublaoui *et al.*, 2008). Postnatal *Sim1* deficiency causes similar phenotypes of the germline *Sim1* deficiency, with hyperphagic obesity and

reduced OT expression (Tolson *et al.*, 2010). The postnatal targeting strategy allowed for the generation of viable conditional *Sim1-/-* mice, which also displayed obesity and hyperphagia. Importantly, both postnatal *Sim1-/- and* +/- mice had a drastic reduction in the expression of the melanocortin 4 receptor (Mc4R) in the PVN (Tolson *et al.*, 2010), again linking the leptin-melanocortin system to obesity in the hypothalamus. Indeed, mutations in the Mc4R are the most common genetic cause of human obesity, accounting for approximately 5% of all cases (Vaisse *et al.*, 2000, Farooqi *et al.*, 2003).

Taken together, the findings presented in this thesis on *Magel2*-null mice are in line with previously described models of obesity with hypothalamic dysfunction, supporting the idea that *Magel2* is impacting energy homeostasis through action in the leptin-melanocortin signaling pathway. Further understanding of leptin-melanocortin signaling is important not only for understanding syndromic and genetic forms of obesity, but also for common diet-induced obesity, which often leads to dysfunction of leptin-melanocortin pathways in the hypothalamus (Horvath *et al.*, 2010)(Enriori *et al.*, 2007).

5.6 Summary of the *Magel2*-null Mouse as a Model for PWS

To date, no single mouse model of PWS has recapitulated the full PWS phenotype, supporting the idea that PWS results from the loss of multiple genes in the chromosome 15q11-q13 region. Some important phenotypes of PWS appear to be present in nearly all mouse models examined, including failure to thrive, aspects of which have been seen in *Ndn*-null mice (Gerard *et al.*, 1999, Muscatelli *et al.*, 2000), *Snord116*-null mice (Skryabin *et al.*, 2007, Ding *et al.*, 2008), and

Magel2-null mice (Bischof *et al.*, 2007, Schaller *et al.*, 2010). Importantly, no mouse model thus far has been able to fully recapitulate the dramatic hyperphagia and obesity seen in PWS, though *Magel2*-null mice do consume an excess level of food based on their energy expenditure, and do develop a moderate degree of obesity (Kozlov *et al.*, 2007a, Bischof *et al.*, 2007). This may be related to species differences between mice and humans, with hedonic and behavioural aspects of human behaviour being especially difficult to model and assess in mice (Crawley., 2007, Crawley., 2008).

The *Magel2*-null mouse has emerged as a strong PWS model by demonstrating a role in numerous PWS phenotypes. Table 5.1 outlines findings in *Magel2*-null mice that relate to PWS, largely based on the PWS diagnostic criteria outlined in Table 1.1. I and others have indeed shown that loss of *Magel2* leads to numerous PWS phenotypes, which are hypothalamic in origin. With notable circadian rhythm defects, energy balance abnormalities, dysregulation of several hypothalamic-pituitary axes, and behavioural abnormalities seen in *Magel2*-null mice, there is strong evidence to support a role for *MAGEL2* in the pathophysiology of PWS.

Despite this phenotypic overlap, *Magel2*-null mice are not a perfect model for PWS. Some of the PWS phenotypes seen in *Magel2*-null mice are less severe than seen in people with PWS. This could be a result of species differences, but is very likely a result of the multiple genes lost in PWS. For example, though *Magel2*-null mice have delayed puberty and early-onset infertility, both male and female mice do present a window of fertility and appear

to have normal gonadal structure. Coupling the defects seen in *Magel2*-null mice with the loss of GnRH neurons and the reduced projections of GnRH neurons to the ME seen in *Ndn*-null mice (Muscatelli *et al.*, 2000, Miller *et al.*, 2009), a more severe reproductive phenotype is predicted. Though they do not develop obesity, *Ndn*-null mice show increased potential for adipose differentiation, and increased adiposity on a high-fat diet (Bush and Wevrick, 2012), which would likely exacerbate the weight gain and adiposity seen in *Magel2*-null mice. Though not conclusively demonstrated, if altered editing of the $5HT_{2C}R$ seen in the IC deletion PWS mouse model is a result of loss of *Snord115* or another PWS gene other than *Magel2*, this could also exacerbate the behavioural and energy balance defects seen in *Magel2*-null mice. Altered $5HT_{2C}R$ editing independent of *Magel2*-loss could also worsen the HPA defects that have been described in *Magel2*-null mice (Tennese and Wevrick, 2011). Other PWS phenotypes including developmental delay, ocular phenotypes, and characteristic facial features have not been observed in *Magel2*-null mice, suggesting that other PWS candidate genes underlie the development of these traits.

Though not a perfect model of PWS, the *Magel2*-null mouse model provides insight into a number of key phenotypes associated with PWS, and also provides further insight to leptin signaling in the hypothalamus, understanding of which is important for understanding mechanisms of obesity in general. Determining a cellular role for Magel2 will help suggest and develop therapeutic avenues for the impaired physiology seen in *Magel2*-null mice, and the translation

of these therapies to people with PWS could lead to significant quality of life improvement for these individuals.

Table 5.1 PWS Phenotypes Recapitulated in *Magel2*-null Mice

PWS Phenotype	*Magel2* -null Mouse Finding	Reference
Neonatal hypotonia and poor suck	Impaired Suckling Adult reduction in lean mass	(Schaller et al., 2010) (Bischof et al., 2007)
Failure to Thrive	Neonatal Lethality Low pre-weaning body weight	(Schaller et al., 2010) (Bischof et al., 2007)
Delayed/Incomplete puberty, Hypogonadism, Infertility	Delayed/extended puberty, early onset infertility	(Mercer and Wevrick, 2009 - Chapter 3)
Hyperphagia and Obesity	Increase energy intake for energy expenditure Adult Obesity with increased adipose mass Leptin Insensitivity	(Kozlov et al., 2007; Bischof et al., 2007) (Bischof et al., 2007; Mercer et al., submitted - Chapter 4) (Mercer et al., submitted - Chapter 4)
Behavioural Problems	Anxious phenotype, avoicance of novel objects	(Mercer et al., 2009 - Chapter 2)
GH Deficiency, short stature	Impaired GH release, Low IGF1-Levels	(Tennese and Wevrick, 2010)
Sleep disturbances, daytime sleepiness	Circadian rhythm defects, fragmented activity	(Kozlov et al., 2007)
Central adrenal insufficency	Dysregulation of Hypothalamic-pituitary-adrenal axis	(Tennese and Wevrick, 2010)
Scoliosis or Kyphosis	Kyphosis	Wevrick Laboratory, unpublished data

5.7 Bibliography

Abramowitz,J. and Birnbaumer,L. (2009) "Physiology and pathophysiology of canonical transient receptor potential channels." FASEB J. **23**(2):297-328.

Abramowitz,J. and Birnbaumer,L. (2007) "Know thy neighbor: a survey of diseases and complex syndromes that map to chromosomal regions encoding TRP channels." Handb.Exp.Pharmacol. **(179)**(179):379-408.

Acuna-Goycolea,C. and van den Pol,A. N. (2005) "Peptide YY(3-36) inhibits both anorexigenic proopiomelanocortin and orexigenic neuropeptide Y neurons: implications for hypothalamic regulation of energy homeostasis." J.Neurosci. **25**(45):10510-10519.

Ahima,R. S. and Lazar,M. A. (2008) "Adipokines and the peripheral and neural control of energy balance." Mol.Endocrinol. **22**(5):1023-1031.

Ahima,R. S., Dushay,J., Flier,S. N., Prabakaran,D. and Flier,J. S. (1997) "Leptin accelerates the onset of puberty in normal female mice." J.Clin.Invest. **99**(3):391-395.

Alba,M., Fintini,D., Sagazio,A., *et al.* (2006) "Once-daily administration of CJC-1295, a long-acting growth hormone-releasing hormone (GHRH) analog, normalizes growth in the GHRH knockout mouse." Am.J.Physiol.Endocrinol.Metab. **291**(6):E1290-4.

Alba,M., Fintini,D. and Salvatori,R. (2005) "Effects of recombinant mouse growth hormone treatment on growth and body composition in GHRH knock out mice." Growth Horm.IGF Res. **15**(4):275-282.

Bachelot,A. and Binart,N. (2007) "Reproductive role of prolactin." Reproduction **133**(2):361-369.

Baumann,H., Morella,K. K., White,D. W., *et al.* (1996) "The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors." Proc.Natl.Acad.Sci.U.S.A. **93**(16):8374-8378.

Bischof,J. M., Stewart,C. L. and Wevrick,R. (2007) "Inactivation of the mouse Magel2 gene results in growth abnormalities similar to Prader-Willi syndrome." Hum.Mol.Genet. **16**(22):2713-2719.

Bjorklund,A. and Dunnett,S. B. (2007) "Dopamine neuron systems in the brain: an update." Trends Neurosci. **30**(5):194-202.

Bolyakov,A. and Paduch,D. A. (2011) "Prolactin in men's health and disease." Curr.Opin.Urol. **21**(6):527-534.

Borrow,A. P. and Cameron,N. M. (2012) "The role of oxytocin in mating and pregnancy." Horm.Behav. **61**(3):266-276.

Bosch,O. J. and Neumann,I. D. (2012) "Both oxytocin and vasopressin are mediators of maternal care and aggression in rodents: From central release to sites of action." Horm.Behav. **61**(3):293-303.

Bush,J. R. and Wevrick,R. (2012) "Loss of the Prader-Willi obesity syndrome protein necdin promotes adipogenesis." Gene **497**(1):45-51.

Butler,M. G., Theodoro,M. F., Bittel,D. C. and Donnelly,J. E. (2007) "Energy expenditure and physical activity in Prader-Willi syndrome: comparison with obese subjects." Am.J.Med.Genet.A. **143**(5):449-459.

Carrel,A. L., Myers,S. E., Whitman,B. Y., Eickhoff,J. and Allen,D. B. (2010) "Long-term growth hormone therapy changes the natural history of body composition and motor function in children with prader-willi syndrome." J.Clin.Endocrinol.Metab. **95**(3):1131-1136.

Chehab,F. F., Mounzih,K., Lu,R. and Lim,M. E. (1997) "Early onset of reproductive function in normal female mice treated with leptin." Science **275**(5296):88-90.

Chehab,F. F., Lim,M. E. and Lu,R. (1996) "Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin." Nat.Genet. **12**(3):318-320.

Clement,K., Vaisse,C., Lahlou,N., *et al.* (1998) "A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction." Nature **392**(6674):398-401.

Coleman,D. L. (2010) "A historical perspective on leptin." Nat.Med. **16**(10):1097-1099.

Cowley,M. A., Smart,J. L., Rubinstein,M., *et al.* (2001) "Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus." Nature **411**(6836):480-484.

Crawley,J. N. (2008) "Behavioral phenotyping strategies for mutant mice." Neuron **57**(6):809-818.

Crawley,J. N. (2007)"Whats's wrong with my mouse?: Behavioral phenotyping of transgenic and knockout mice"John Wiley & Sons IncHoboken, NY.

de Lind van Wijngaarden,R. F., Siemensma,E. P., Festen,D. A., *et al.* (2009) "Efficacy and safety of long-term continuous growth hormone treatment in

children with Prader-Willi syndrome." J.Clin.Endocrinol.Metab. **94**(11):4205-4215.

Ding,F., Li,H. H., Zhang,S., *et al.* (2008) "SnoRNA Snord116 (Pwcr1/MBII-85) deletion causes growth deficiency and hyperphagia in mice." PLoS One **3**(3):e1709.

Doe,C. M., Relkovic,D., Garfield,A. S., *et al.* (2009) "Loss of the imprinted snoRNA mbii-52 leads to increased 5htr2c pre-RNA editing and altered 5HT2CR-mediated behaviour." Hum.Mol.Genet. **18**(12):2140-2148.

El-Haschimi,K., Pierroz,D. D., Hileman,S. M., Bjorbaek,C. and Flier,J. S. (2000) "Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity." J.Clin.Invest. **105**(12):1827-1832.

Elmquist,J. K., Bjorbaek,C., Ahima,R. S., Flier,J. S. and Saper,C. B. (1998) "Distributions of leptin receptor mRNA isoforms in the rat brain." J.Comp.Neurol. **395**(4):535-547.

Enriori,P. J., Evans,A. E., Sinnayah,P., *et al.* (2007) "Diet-induced obesity causes severe but reversible leptin resistance in arcuate melanocortin neurons." Cell.Metab. **5**(3):181-194.

Ershov,P. V., Ugrumov,M. V., Calas,A., Makarenko,I. G., Krieger,M. and Thibault,J. (2002) "Neurons possessing enzymes of dopamine synthesis in the mediobasal hypothalamus of rats. Topographic relations and axonal projections to the median eminence in ontogenesis." J.Chem.Neuroanat. **24**(2):95-107.

Farooqi,I. S., Wangensteen,T., Collins,S., *et al.* (2007) "Clinical and molecular genetic spectrum of congenital deficiency of the leptin receptor." N.Engl.J.Med. **356**(3):237-247.

Farooqi,I. S., Keogh,J. M., Yeo,G. S., Lank,E. J., Cheetham,T. and O'Rahilly,S. (2003) "Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene." N.Engl.J.Med. **348**(12):1085-1095.

Fei,H., Okano,H. J., Li,C., *et al.* (1997) "Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues." Proc.Natl.Acad.Sci.U.S.A. **94**(13):7001-7005.

Fetissov,S. O., Meguid,M. M., Sato,T. and Zhang,L. H. (2002) "Expression of dopaminergic receptors in the hypothalamus of lean and obese Zucker rats and food intake." Am.J.Physiol.Regul.Integr.Comp.Physiol. **283**(4):R905-10.

Frisch,R. E. and McArthur,J. W. (1974) "Menstrual cycles: fatness as a determinant of minimum weight for height necessary for their maintenance or onset." Science **185**(4155):949-951.

Garcia-Tornadu,I., Risso,G., Perez-Millan,M. I., *et al.* (2010) "Neurotransmitter modulation of the GHRH-GH axis." Front.Horm.Res. **38**:59-69.

Gerard,M., Hernandez,L., Wevrick,R. and Stewart,C. L. (1999) "Disruption of the mouse necdin gene results in early post-natal lethality." Nat.Genet. **23**(2):199-202.

Ghamari-Langroudi,M., Colmers,W. F. and Cone,R. D. (2005) "PYY3-36 inhibits the action potential firing activity of POMC neurons of arcuate nucleus through postsynaptic Y2 receptors." Cell.Metab. **2**(3):191-199.

Gibson,W. T., Farooqi,I. S., Moreau,M., *et al.* (2004) "Congenital leptin deficiency due to homozygosity for the Delta133G mutation: report of another case and evaluation of response to four years of leptin therapy." J.Clin.Endocrinol.Metab. **89**(10):4821-4826.

Goldman,J. M., Murr,A. S. and Cooper,R. L. (2007) "The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies." Birth Defects Res.B.Dev.Reprod.Toxicol. **80**(2):84-97.

Gosden,R. G., Laing,S. C., Felicio,L. S., Nelson,J. F. and Finch,C. E. (1983) "Imminent oocyte exhaustion and reduced follicular recruitment mark the transition to acyclicity in aging C57BL/6J mice." Biol.Reprod. **28**(2):255-260.

Greenfield,J. R. (2011) "Melanocortin signalling and the regulation of blood pressure in human obesity." J.Neuroendocrinol. **23**(2):186-193.

Guo,D. F. and Rahmouni,K. (2011) "Molecular basis of the obesity associated with Bardet-Biedl syndrome." Trends Endocrinol.Metab. **22**(7):286-293.

Han,S. K., Gottsch,M. L., Lee,K. J., *et al.* (2005) "Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty." J.Neurosci. **25**(49):11349-11356.

Harvey,J. and Ashford,M. L. (2003) "Leptin in the CNS: much more than a satiety signal." Neuropharmacology **44**(7):845-854.

Hashimoto,T., Mori,K., Yoneda,Y., *et al.* (1998) "Proton magnetic resonance spectroscopy of the brain in patients with Prader-Willi syndrome." Pediatr.Neurol. **18**(1):30-35.

Hayano,F., Nakamura,M., Asami,T., *et al.* (2009) "Smaller amygdala is associated with anxiety in patients with panic disorder." Psychiatry Clin.Neurosci. **63**(3):266-276.

Heisler,L. K., Pronchuk,N., Nonogaki,K., *et al.* (2007) "Serotonin activates the hypothalamic-pituitary-adrenal axis via serotonin 2C receptor stimulation." J.Neurosci. **27**(26):6956-6964.

Heisler,L. K., Jobst,E. E., Sutton,G. M., *et al.* (2006) "Serotonin reciprocally regulates melanocortin neurons to modulate food intake." Neuron **51**(2):239-249.

Heisler,L. K., Cowley,M. A., Kishi,T., *et al.* (2003) "Central serotonin and melanocortin pathways regulating energy homeostasis." Ann.N.Y.Acad.Sci. **994**:169-174.

Hellings,J. A. and Warnock,J. K. (1994) "Self-injurious behavior and serotonin in Prader-Willi syndrome." Psychopharmacol.Bull. **30**(2):245-250.

Hohmann,C. F., Walker,E. M., Boylan,C. B. and Blue,M. E. (2007) "Neonatal serotonin depletion alters behavioral responses to spatial change and novelty." Brain Res. **1139**:163-177.

Horvath,T. L., Sarman,B., Garcia-Caceres,C., *et al.* (2010) "Synaptic input organization of the melanocortin system predicts diet-induced hypothalamic reactive gliosis and obesity." Proc.Natl.Acad.Sci.U.S.A. **107**(33):14875-14880.

Hoybye,C. (2004) "Endocrine and metabolic aspects of adult Prader-Willi syndrome with special emphasis on the effect of growth hormone treatment." Growth Horm.IGF Res. **14**(1):1-15.

Hummel,K. P., Dickie,M. M. and Coleman,D. L. (1966) "Diabetes, a new mutation in the mouse." Science **153**(3740):1127-1128.

Ingalls,A. M., DICKIE,M. M. and SNELL,G. D. (1950) "Obese, a new mutation in the house mouse." J.Hered. **41**(12):317-318.

Irwig,M. S., Fraley,G. S., Smith,J. T., *et al.* (2004) "Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat." Neuroendocrinology **80**(4):264-272.

Kennedy,G. C. (1969) "Interactions between feeding behavior and hormones during growth." Ann.N.Y.Acad.Sci. **157**(2):1049-1061.

Kishore,S., Khanna,A., Zhang,Z., *et al.* (2010) "The snoRNA MBII-52 (SNORD 115) is processed into smaller RNAs and regulates alternative splicing." Hum.Mol.Genet. **19**(7):1153-1164.

Kishore,S. and Stamm,S. (2006) "The snoRNA HBII-52 regulates alternative splicing of the serotonin receptor 2C." Science **311**(5758):230-232.

Korenbrot,C. C., Huhtaniemi,I. T. and Weiner,R. I. (1977) "Preputial separation as an external sign of pubertal development in the male rat." Biol.Reprod. **17**(2):298-303.

Kozlov,S. V., Bogenpohl,J. W., Howell,M. P., *et al.* (2007) "The imprinted gene Magel2 regulates normal circadian output." Nat.Genet. **39**(10):1266-1272.

Kublaoui,B. M., Gemelli,T., Tolson,K. P., Wang,Y. and Zinn,A. R. (2008) "Oxytocin deficiency mediates hyperphagic obesity of Sim1 haploinsufficient mice." Mol.Endocrinol. **22**(7):1723-1734.

Lam,D. D., Przydzial,M. J., Ridley,S. H., *et al.* (2008) "Serotonin 5-HT2C receptor agonist promotes hypophagia via downstream activation of melanocortin 4 receptors." Endocrinology **149**(3):1323-1328.

Lamberts,S. W. and Macleod,R. M. (1990) "Regulation of prolactin secretion at the level of the lactotroph." Physiol.Rev. **70**(2):279-318.

Lee,G. H., Proenca,R., Montez,J. M., *et al.* (1996) "Abnormal splicing of the leptin receptor in diabetic mice." Nature **379**(6566):632-635.

Lee,S., Walker,C. L., Karten,B., *et al.* (2005) "Essential role for the Prader-Willi syndrome protein necdin in axonal outgrowth." Hum.Mol.Genet. **14**(5):627-637.

Leshan,R. L., Bjornholm,M., Munzberg,H. and Myers,M. G.,Jr. (2006) "Leptin receptor signaling and action in the central nervous system." Obesity (Silver Spring) **14 Suppl 5**:208S-212S.

Li,G., Zhang,Y., Wilsey,J. T. and Scarpace,P. J. (2004) "Unabated anorexic and enhanced thermogenic responses to melanotan II in diet-induced obese rats despite reduced melanocortin 3 and 4 receptor expression." J.Endocrinol. **182**(1):123-132.

Lin,S., Thomas,T. C., Storlien,L. H. and Huang,X. F. (2000) "Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice." Int.J.Obes.Relat.Metab.Disord. **24**(5):639-646.

Mandiyan,V. S., Coats,J. K. and Shah,N. M. (2005) "Deficits in sexual and aggressive behaviors in Cnga2 mutant mice." Nat.Neurosci. **8**(12):1660-1662.

Marsh,D. J., Hollopeter,G., Huszar,D., *et al.* (1999) "Response of melanocortin-4 receptor-deficient mice to anorectic and orexigenic peptides." Nat.Genet. **21**(1):119-122.

Meguid,M. M., Yang,Z. J. and Laviano,A. (1997) "Meal size and number: relationship to dopamine levels in the ventromedial hypothalamic nucleus." Am.J.Physiol. **272**(6 Pt 2):R1925-30.

Meguid,M. M., Yang,Z. J. and Koseki,M. (1995) "Eating induced rise in LHA-dopamine correlates with meal size in normal and bulbectomized rats." Brain Res.Bull. **36**(5):487-490.

Mercer,J. G., Hoggard,N., Williams,L. M., Lawrence,C. B., Hannah,L. T. and Trayhurn,P. (1996) "Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization." FEBS Lett. **387**(2-3):113-116.

Messager,S., Chatzidaki,E. E., Ma,D., *et al.* (2005) "Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54." Proc.Natl.Acad.Sci.U.S.A. **102**(5):1761-1766.

Michaud,J. L., Boucher,F., Melnyk,A., *et al.* (2001) "Sim1 haploinsufficiency causes hyperphagia, obesity and reduction of the paraventricular nucleus of the hypothalamus." Hum.Mol.Genet. **10**(14):1465-1473.

Michaud,J. L., Rosenquist,T., May,N. R. and Fan,C. M. (1998) "Development of neuroendocrine lineages requires the bHLH-PAS transcription factor SIM1." Genes Dev. **12**(20):3264-3275.

Miller,B. H., Olson,S. L., Turek,F. W., Levine,J. E., Horton,T. H. and Takahashi,J. S. (2004) "Circadian clock mutation disrupts estrous cyclicity and maintenance of pregnancy." Curr.Biol. **14**(15):1367-1373.

Miller,J. L., Couch,J. A., Leonard,C. M., *et al.* (2007a) "Sylvian fissure morphology in Prader-Willi syndrome and early-onset morbid obesity." Genet.Med. **9**(8):536-543.

Miller,J. L., Couch,J. A., Schmalfuss,I., He,G., Liu,Y. and Driscoll,D. J. (2007b) "Intracranial abnormalities detected by three-dimensional magnetic resonance imaging in Prader-Willi syndrome." Am.J.Med.Genet.A. **143**(5):476-483.

Miller,N. L., Wevrick,R. and Mellon,P. L. (2009) "Necdin, a Prader-Willi syndrome candidate gene, regulates gonadotropin-releasing hormone neurons during development." Hum.Mol.Genet. **18**(2):248-260.

Miller,N. L., Wevrick,R. and Mellon,P. L. (2008) "Necdin, a Prader-Willi Syndrome candidate gene, regulates gonadotropin-releasing hormone neurons during development." Hum.Mol.Genet.

Montague,C. T., Farooqi,I. S., Whitehead,J. P., *et al.* (1997) "Congenital leptin deficiency is associated with severe early-onset obesity in humans." Nature **387**(6636):903-908.

Morabito,M. V., Abbas,A. I., Hood,J. L., *et al.* (2010) "Mice with altered serotonin 2C receptor RNA editing display characteristics of Prader-Willi syndrome." Neurobiol.Dis. **39**(2):169-180.

Muscatelli,F., Abrous,D. N., Massacrier,A., *et al.* (2000) "Disruption of the mouse Necdin gene results in hypothalamic and behavioral alterations reminiscent of the human Prader-Willi syndrome." Hum.Mol.Genet. **9**(20):3101-3110.

Pierroz,D. D., Ziotopoulou,M., Ungsunan,L., Moschos,S., Flier,J. S. and Mantzoros,C. S. (2002) "Effects of acute and chronic administration of the melanocortin agonist MTII in mice with diet-induced obesity." Diabetes **51**(5):1337-1345.

Portillo,W. and Paredes,R. G. (2004) "Sexual incentive motivation, olfactory preference, and activation of the vomeronasal projection pathway by sexually relevant cues in non-copulating and naive male rats." Horm.Behav. **46**(3):330-340.

Prpic,V., Watson,P. M., Frampton,I. C., Sabol,M. A., Jezek,G. E. and Gettys,T. W. (2003) "Differential mechanisms and development of leptin resistance in A/J versus C57BL/6J mice during diet-induced obesity." Endocrinology **144**(4):1155-1163.

Qiu,J., Fang,Y., Bosch,M. A., Ronnekleiv,O. K. and Kelly,M. J. (2011) "Guinea pig kisspeptin neurons are depolarized by leptin via activation of TRPC channels." Endocrinology **152**(4):1503-1514.

Qiu,J., Fang,Y., Ronnekleiv,O. K. and Kelly,M. J. (2010) "Leptin excites proopiomelanocortin neurons via activation of TRPC channels." J.Neurosci. **30**(4):1560-1565.

Rahmouni,K., Fath,M. A., Seo,S., *et al.* (2008) "Leptin resistance contributes to obesity and hypertension in mouse models of Bardet-Biedl syndrome." J.Clin.Invest. **118**(4):1458-1467.

Rau,H., Reaves,B. J., O'Rahilly,S. and Whitehead,J. P. (1999) "Truncated human leptin (delta133) associated with extreme obesity undergoes proteasomal degradation after defective intracellular transport." Endocrinology **140**(4):1718-1723.

Reus,L., Zwarts,M., van Vlimmeren,L. A., Willemsen,M. A., Otten,B. J. and Nijhuis-van der Sanden,M. W. (2011) "Motor problems in Prader-Willi

syndrome: a systematic review on body composition and neuromuscular functioning." Neurosci.Biobehav.Rev. **35**(3):956-969.

Riccio,A., Medhurst,A. D., Mattei,C., *et al.* (2002) "mRNA distribution analysis of human TRPC family in CNS and peripheral tissues." Brain Res.Mol.Brain Res. **109**(1-2):95-104.

Roseberry,A. G., Liu,H., Jackson,A. C., Cai,X. and Friedman,J. M. (2004) "Neuropeptide Y-mediated inhibition of proopiomelanocortin neurons in the arcuate nucleus shows enhanced desensitization in ob/ob mice." Neuron **41**(5):711-722.

Safranski,T. J., Lamberson,W. R. and Keisler,D. H. (1993) "Correlations among three measures of puberty in mice and relationships with estradiol concentration and ovulation." Biol.Reprod. **48**(3):669-673.

Sanchez-Ortiga,R., Klibanski,A. and Tritos,N. A. (2011) "Effects of Recombinant Human Growth Hormone Therapy in Adults with Prader-Willi Syndrome: A Meta-Analysis." Clin.Endocrinol.(Oxf)

Schaller,F., Watrin,F., Sturny,R., Massacrier,A., Szepetowski,P. and Muscatelli,F. (2010) "A single postnatal injection of oxytocin rescues the lethal feeding behaviour in mouse newborns deficient for the imprinted Magel2 gene." Hum.Mol.Genet. **19**(24):4895-4905.

Seo,S., Guo,D. F., Bugge,K., Morgan,D. A., Rahmouni,K. and Sheffield,V. C. (2009) "Requirement of Bardet-Biedl syndrome proteins for leptin receptor signaling." Hum.Mol.Genet. **18**(7):1323-1331.

Siemensma,E. P., Tummers-de Lind van Wijngaarden,R. F., Festen,D. A., *et al.* (2012) "Beneficial Effects of Growth Hormone Treatment on Cognition in Children with Prader-Willi Syndrome: A Randomized Controlled Trial and Longitudinal Study." J.Clin.Endocrinol.Metab.

Skryabin,B. V., Gubar,L. V., Seeger,B., *et al.* (2007) "Deletion of the MBII-85 snoRNA gene cluster in mice results in postnatal growth retardation." PLoS Genet. **3**(12):e235.

Spergel,D. J., Kruth,U., Hanley,D. F., Sprengel,R. and Seeburg,P. H. (1999) "GABA- and glutamate-activated channels in green fluorescent protein-tagged gonadotropin-releasing hormone neurons in transgenic mice." J.Neurosci. **19**(6):2037-2050.

Strader,A. D., Shi,H., Ogawa,R., Seeley,R. J. and Reizes,O. (2007) "The effects of the melanocortin agonist (MT-II) on subcutaneous and visceral adipose tissue in rodents." J.Pharmacol.Exp.Ther. **322**(3):1153-1161.

Swaab,D. F., Purba,J. S. and Hofman,M. A. (1995) "Alterations in the hypothalamic paraventricular nucleus and its oxytocin neurons (putative satiety cells) in Prader-Willi syndrome: a study of five cases." J.Clin.Endocrinol.Metab. **80**(2):573-579.

Takano,H. and Abe,K. (1987) "Age-related histologic changes in the adult mouse testis." Arch.Histol.Jpn. **50**(5):533-544.

Tauber,M., Mantoulan,C., Copet,P., *et al.* (2011) "Oxytocin may be useful to increase trust in others and decrease disruptive behaviours in patients with Prader-Willi syndrome: a randomised placebo-controlled trial in 24 patients." Orphanet J.Rare Dis. **6**:47.

Tennese,A. A. and Wevrick,R. (2011) "Impaired hypothalamic regulation of endocrine function and delayed counterregulatory response to hypoglycemia in Magel2-null mice." Endocrinology **152**(3):967-978.

Tolson,K. P., Gemelli,T., Gautron,L., Elmquist,J. K., Zinn,A. R. and Kublaoui,B. M. (2010) "Postnatal Sim1 deficiency causes hyperphagic obesity and reduced Mc4r and oxytocin expression." J.Neurosci. **30**(10):3803-3812.

Vaisse,C., Clement,K., Durand,E., Hercberg,S., Guy-Grand,B. and Froguel,P. (2000) "Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity." J.Clin.Invest. **106**(2):253-262.

van den Pol,A. N., Herbst,R. S. and Powell,J. F. (1984) "Tyrosine hydroxylase-immunoreactive neurons of the hypothalamus: a light and electron microscopic study." Neuroscience **13**(4):1117-1156.

van den Top,M., Lee,K., Whyment,A. D., Blanks,A. M. and Spanswick,D. (2004) "Orexigen-sensitive NPY/AgRP pacemaker neurons in the hypothalamic arcuate nucleus." Nat.Neurosci. **7**(5):493-494.

Vandenbergh,J. G. (1973) "Effects of central and peripheral anosmia on reproduction of female mice." Physiol.Behav. **10**(2):257-261.

Waldherr,M. and Neumann,I. D. (2007) "Centrally released oxytocin mediates mating-induced anxiolysis in male rats." Proc.Natl.Acad.Sci.U.S.A. **104**(42):16681-16684.

Wang,L., Simpson,H. B. and Dulawa,S. C. (2009) "Assessing the validity of current mouse genetic models of obsessive-compulsive disorder." Behav.Pharmacol. **20**(2):119-133.

Wang,Z., Nudelman,A. and Storm,D. R. (2007) "Are pheromones detected through the main olfactory epithelium?" Mol.Neurobiol. **35**(3):317-323.

Warnock,J. K., Clayton,A. H., Shaw,H. A. and O'Donnell,T. (1995) "Onset of menses in two adult patients with Prader-Willi syndrome treated with fluoxetine." Psychopharmacol.Bull. **31**(2):239-242.

Warnock,J. K. and Kestenbaum,T. (1992) "Pharmacologic treatment of severe skin-picking behaviors in Prader-Willi syndrome. Two case reports." Arch.Dermatol. **128**(12):1623-1625.

Warren,E. J., Allen,C. N., Brown,R. L. and Robinson,D. W. (2006) "The light-activated signaling pathway in SCN-projecting rat retinal ganglion cells." Eur.J.Neurosci. **23**(9):2477-2487.

Whitman,B. Y., Myers,S., Carrel,A. and Allen,D. (2002) "The behavioral impact of growth hormone treatment for children and adolescents with Prader-Willi syndrome: a 2-year, controlled study." Pediatrics **109**(2):E35.

Williams,K. W., Sohn,J. W., Donato,J.,Jr, *et al.* (2011) "The acute effects of leptin require PI3K signaling in the hypothalamic ventral premammillary nucleus." J.Neurosci. **31**(37):13147-13156.

Yoon,H., Enquist,L. W. and Dulac,C. (2005) "Olfactory inputs to hypothalamic neurons controlling reproduction and fertility." Cell **123**(4):669-682.

Zaghloul,N. A. and Katsanis,N. (2009) "Mechanistic insights into Bardet-Biedl syndrome, a model ciliopathy." J.Clin.Invest. **119**(3):428-437.

Zhang,C., Roepke,T. A., Kelly,M. J. and Ronnekleiv,O. K. (2008) "Kisspeptin depolarizes gonadotropin-releasing hormone neurons through activation of TRPC-like cationic channels." J.Neurosci. **28**(17):4423-4434.

Zhang,Y., Collazo,R., Gao,Y., Li,G. and Scarpace,P. J. (2010) "Intermittent MTII application evokes repeated anorexia and robust fat and weight loss." Peptides **31**(4):639-643.