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Role of the Prader-Willi syndrome proteins necdin and Magel2 in the nervous system

by

Alysa Anne Tennese

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Examining committee

Dr. Rachel Wevrick, Department of Medical Genetics

Dr. John Greer, Department of Physiology

Dr. Andrew Waskiewicz, Department of Biological Sciences

Dr. Stacey Hume, Department of Medical Genetics

Dr. Keith Sharkey, Department of Physiology and Pharmacology, University of Calgary

Abstract

Prader-Willi syndrome (PWS) is a rare, neurodevelopmental disorder with multiple features caused by hypothalamic deficiency, including infantile failure to thrive, hyperphagia leading to obesity, growth hormone deficiency, hypogonadism, and central adrenal insufficiency. Other features of PWS including global developmental delay, hypotonia, pain insensitivity, gastrointestinal dysfunction, and psychiatric disorders are caused by deficits in other regions of the nervous system. PWS is caused by the loss of a subset of paternally-expressed genes on chromosome 15, which includes *NDN* and *MAGEL2*. Necdin and Magel2 are both members of the melanoma antigen (MAGE) family of proteins and are expressed throughout development, particularly in the nervous system. This thesis describes experiments that examine the loss of function of necdin and Magel2 in mice and their potential roles in the pathogenesis of PWS.

Targeted inactivation of *Ndn* and *Magel2* in mice has aided in determining how loss of function of these proteins affects the development and function of the nervous system. Loss of necdin causes reduced axonal outgrowth and neuronal differentiation in the central and peripheral sensory nervous systems. I examined the autonomic nervous system in *Ndn*-null embryos and identified a defect in the migration of the most rostral sympathetic chain ganglion and consequently increased neuronal cell death and reduced innervation of target tissues supplied by this ganglion. Reduced axonal outgrowth was observed throughout the sympathetic nervous system in *Ndn*-null embryos although no gross deficits in the

parasympathetic and enteric nervous systems were identified. Loss of Magel2 causes reduced fertility and abnormal circadian rhythm patterns in mice. I further identified an altered response to stress, a delayed response to insulin-induced hypoglycemia, a reduced stimulated growth hormone response, and lower thyroid hormone levels in *Magel2*-null mice, indicative of deficits in multiple hypothalamic-pituitary axes. The findings presented in this thesis support a role for necdin and Magel2 in the development and function of the nervous system. The data also indicates that these MAGE proteins play a key role in multiple features of PWS, including endocrine deficiencies and autonomic dysfunction.

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List of Abbreviations

ACTH	adrenocorticotropic hormone
ANS	autonomic nervous system
AS	Angelman syndrome
AVP	arginine vasopressin
BBS	Bardet-Biedl syndrome
BDNF	brain-derived neurotrophic factor
BMP	bone morphogenetic protein
ChAT	choline acetyltransferase
CRH	corticotropin-releasing hormone
DEPC	diethyl pyrocarbonate
DIG	digoxygenin
DRG	dorsal root ganglion
ENS	enteric nervous system
FSH	follicle stimulating hormone
GDNF	glial cell-derived neurotrophic factor
GH	growth hormone
GHRH	growth hormone-releasing hormone
GI	gastrointestinal
GnRH	gonadotropin-releasing hormone
GTT	glucose tolerance test

HPA	hypothalamic-pituitary-adrenal
HMW	high molecular weight
HOMA-IR	homeostasis model assessment of insulin resistance
IC	imprinting centre
IGF	insulin-like growth factor
IHC	immunohistochemistry
ITT	insulin tolerance test
LH	luteinizing hormone
MAGE	melanoma antigen
MHD	MAGE homology domain
NGF	nerve growth factor
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with 0.1% Tween
PFA	paraformaldehyde in phosphate-buffered saline
PWS	Prader-Willi syndrome
RNA	ribonucleic acid
RT	room temperature
SCG	superior cervical ganglia
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean

snoRNA	small nucleolar RNA
SSC	saline-sodium citrate
T_3	3,5,3’-triiodothyronine
T_4	thyroxine
TH	tyrosine hydroxylase
TRH	thyrotropin-releasing hormone
Trk	tropomyosin-related kinase
TSH	thyroid stimulating hormone

Chapter 1. Introduction

1.1 Prader-Willi syndrome (PWS)

1.1.1 Clinical features of PWS

Prader-Willi syndrome (PWS) is a neurodevelopmental disorder that was first described in 1956 and occurs in approximately 1:15,000 live births (Prader 1956). PWS occurs equally among males and females, and occurs in all races. Major diagnostic criteria for PWS include hypotonia and feeding difficulties in the neonatal period, global developmental delay, and hypogonadism which are observed in greater than 90% of all PWS individuals (Table 1-1) (Butler 1990; Holm et al. 1993). The remaining major criteria include failure to thrive in infancy, hyperphagia, excessive weight gain beginning in early childhood, and craniofacial abnormalities (Table 1-1) (Holm et al. 1993). Hypogonadism can be identified in younger male patients with cryptorchidism and/or smaller penises but is difficult to assess in neonatal PWS females (Crino et al. 2003; Hoybye et al. 2003; Eiholzer et al. 2006). PWS individuals often have delayed or absent puberty. Throughout the natural history of PWS, global developmental delay is observed, which results in milestone delays in early childhood with progression to cognitive impairment in adolescence and adulthood.

Minor features in PWS include decreased fetal movements, short stature, small hands and feet, behavioural problems, excessive daytime sleepiness and central or obstructive sleep apneas, hypopigmentation, decreased salivary output with increased viscosity, speech articulation deficits and eye abnormalities as a consequence of persistent hypotonia, and skin picking (Table 1-1) (Holm et al. 1993). Behavioural aspects of PWS include temper tantrums, lying and stealing, and obsessive compulsive tendencies, especially when these behaviours are related to food. Supportive criteria indicate characteristics of PWS that are present in many PWS individuals but are not required for a diagnosis, though they increase the likelihood of a diagnosis of PWS. These features include increased pain threshold, decreased vomiting, scoliosis, adrenal insufficiency causing a reduced response to illness and stress (de Lind van Wijngaarden et al. 2008), early adrenarche, normal muscular studies for the degree of hypotonia, unusual skill

with jigsaw puzzles, ineffective thermoregulation, osteoporosis, and epilepsy (Vendrame et al. 2010) (Table 1-1).

Table 1-1. Diagnostic criteria for Prader-Willi syndrome.

Major criteria (1 point each):
Central neonatal hypotonia
Failure to thrive which may include feeding difficulties
Hyperphagia leading to obesity if food intake is not controlled
Excessive or rapid weight gain for height beginning between ages 1-6
Hypogonadotropic hypogonadism
Global developmental delay
Characteristic craniofacial abnormalities including narrow bifrontal diameter, low-set ears, almond-shaped and down-slanted eyes
Minor criteria (half a point each):
Decreased fetal movements
Short stature
Small hands and feet
Characteristic behavioural problems including obsessive compulsive disorder, temper tantrums, lying and stealing, especially when behaviours are related to food
Hypopigmentation
Sleep disturbance or apnea
Eye abnormalities
Thick viscous saliva
Speech articulation defects
Skin picking
Supportive criteria (no points but strengthen likelihood of diagnosis):
Increased pain threshold
Decreased vomiting
Scoliosis or kyphosis
Adrenal insufficiency
Normal muscular studies for hypotonia
Unusual skill with jigsaw puzzles
Ineffective thermoregulation
Early adrenarche
Osteoporosis
Epilepsy

Note: Diagnosis requires five points in children <3 years with four points being from major criteria and eight points in children >3 years with at least five points being from major criteria. [Adapted from (Holm et al. 1993)].

As clinical features of PWS vary with age, molecular testing criteria have been proposed based on patient age at time of suspected diagnosis (Table 1-2) (Gunay-Aygun et al. 2001; Goldstone et al. 2008). The initial neonatal criteria describe the hypotonic phase of PWS, while the hyperphagic phase begins during the toddler years and progresses into early childhood. How the transition between these two opposite states in energy balance occurs is currently not known, although one study identified clinical obesity in PWS children before the onset of hyperphagia, which suggests an underlying metabolic deficit independent of appetite control (Butler et al. 2009). One theory is that a defect in fetal development causes the neonatal phenotype while the adult phenotype is caused by inadequate compensatory mechanisms, likely hypothalamic in nature (Stefan et al. 2005).

Currently the only treatment available for PWS is growth hormone (GH) therapy which improves body composition, resolves some but not all of the persistent hypotonia, and causes a significant increase in height by increasing growth velocity (Festen et al. 2008; Carrel et al. 2010). Unfortunately, there is no treatment to reduce the insatiable hunger in PWS individuals, which is the feature that most parents would like to have resolved. The main goal with respect to hyperphagic tendencies is to prevent the development of obesity and is accomplished through strict environmental control (Goldstone et al. 2008). Sex hormone replacement and induction of puberty in PWS remains controversial but has been recommended: 1) to improve bone mineral density as PWS individuals are prone to developing osteoporosis; 2) to improve secondary sexual development which benefits mental, emotional and physical well-being; 3) and to maintain muscle mass.

Table 1-2. Suggested Criteria to Prompt DNA Testing for PWS.

Age at Assessment	Features Sufficient to Prompt DNA Testing
Birth to 2 years	Hypotonia with poor suck
2 –6 years	Hypotonia with history of poor suck
	Global developmental delay
	Short stature and/or growth failure associated with accelerated weight gain
6 –12 years	History of hypotonia with poor suck (hypotonia often persists)
	Global developmental delay
	Hyperphagia with central obesity if uncontrolled
13 years through adulthood	Cognitive impairment; usually mild mental retardation
	Hyperphagia with central obesity if uncontrolled
	Hypothalamic hypogonadism and/or typical problems (including temper tantrums and obsessive-compulsive features)

[Adapted from (Gunay-Aygun et al. 2001; Goldstone 2004)]

1.1.2 Genetics of PWS

PWS is caused by the loss of paternally expressed genes on chromosome 15q11-13 (Butler 1990) (Figure 1-1). This region of chromosome 15 is differentially imprinted meaning that genes are subject to different methylation patterns on the paternal and maternal chromosomes resulting in monoallelic rather than biallelic expression (Driscoll et al. 1992). PWS is caused by a microdeletion of the paternally inherited region on chromosome 15q11-13 in 70% of individuals (Ledbetter et al. 1981; Butler and Palmer 1983; Knoll et al. 1989), while 20-25% of PWS individuals inherit two maternal copies of chromosome 15 (Nicholls et al. 1989; Cassidy et al. 1992), and the final 5-10% of patients have an imprinting center deletion or a translocation leading to the disruption and altered methylation of the paternal copy of 15q11-13 (Sutcliffe et al. 1994; Buiting et al. 1995). Loss of expression of five protein-coding genes: *MKRN3*, *NDN*, *MAGEL2*, *C15ORF2*, and *SNURF-SNRPN*, and several small nucleolar RNA (snoRNA) C/D box gene clusters, including *SNORD115/HBII-52* and *SNORD116/HBII-85/PWCR1*, occurs on the paternally-inherited chromosome in PWS individuals. Angelman syndrome (AS) is a separate neurodevelopmental disorder caused by the loss of the maternally-expressed gene, UBE3A on chromosome 15q11-13 (Knoll et al. 1989; Kishino et al. 1997; Matsuura et al. 1997; Sutcliffe et al. 1997)

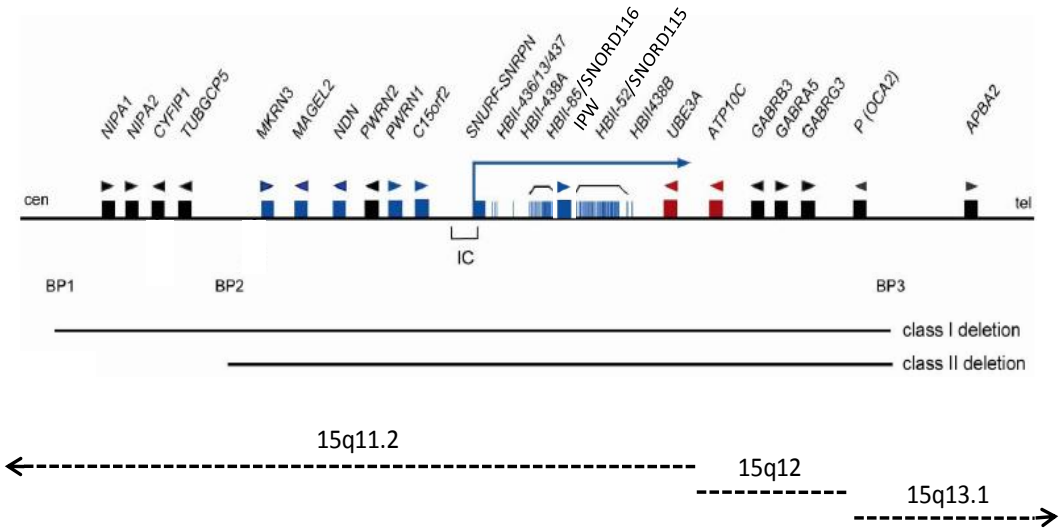

Figure 1-1. Organization of the Prader-Willi/Angelman region on human chromosome 15.

Gene names in blue are paternally expressed and their expression is absent in PWS. Gene names in red are maternally expressed and their expression is absent in AS. Gene names in black are biallelicly expressed. IC, imprinting center. BP refers to breakpoint, which are regions where chromosome rearrangements are most likely to occur, causing deletions in this region on chromosome 15q. [Adapted from (Ramsden et al. 2010)]

PWS candidate genes

NDN and *MAGEL2* belong to the melanoma antigen (MAGE) family of proteins, which all possess a MAGE homology domain (MHD) that is involved in protein-protein interactions (Figure 1-2) (Barker and Salehi 2002). The *NDN* gene codes for necdin. Necdin has a role in terminal differentiation of neurons which is supported by studies showing that PC12 cells transfected with necdin have increased differentiation consequent to increased signaling via $p75^{NTR}$ and tropomyosin-related kinase A (TrkA) (Tcherpakov et al. 2002) and that repression of necdin in embryonic dorsal root ganglia (DRG) suppresses their differentiation (Takazaki et al. 2002). Necdin also induces neurite outgrowth in neuroblastoma cells (Kobayashi et al. 2002) and accelerates neurite outgrowth in transiently transfected PC12 cells (Tcherpakov et al. 2002). Interaction of necdin with proteins occurs via its MHD. Necdin-interacting proteins can be classified as cell cycle regulators, transmembrane receptors, transcription factors, or transcriptional co-regulators and include: E2F1, p53, NEFA, $p75^{NTR}$, hnRNPU, MAGED1, HIF1α, EID1, Sirtuin1, and Nogo-A (Taniura et al. 1998; Taniura et al. 1999; Taniguchi et al. 2000; Taniura and Yoshikawa 2002; Tcherpakov et al. 2002; Kuwajima et al. 2004; Friedman and Fan 2007; Bush and Wevrick 2008; Hasegawa and Yoshikawa 2008; Liu et al. 2009). Necdin also interacts with proteins associated with other monogenic obesity syndromes including Bardet-Biedl syndrome (BBS) proteins BBS4 and BBS8 (Lee et al. 2005), and ARNT2, the dimerization partner of SIM1, whose heterozygous deletion causes a Prader-Willi-like phenotype with excessive hyperphagia (Friedman and Fan 2007).

Like *NDN*, *MAGEL2* is an intronless gene with a MHD involved in protein-protein interactions. Magel2 also interacts with Bardet-Biedl syndrome proteins BBS4 and BBS8, and like necdin, interacts with fasciculation and elongation protein zeta 1 (FEZ1), which implies a role for Magel2 in neuronal development, as is observed with necdin (Lee et al. 2005). *MAGEL2* is expressed throughout the nervous system but is most highly expressed in the hypothalamus, preferentially in the suprachiasmatic and supraoptic nuclei (Lee et al. 2003).

Compared to *MAGEL2*, *NDN* is ubiquitously expressed and is found in both central and peripheral neurons; and skeletal and smooth muscle cells, although its highest expression is in the hindbrain and hypothalamus. Further studies in *Ndn*- and *Magel2*-null mouse models delineating the roles of these MAGE proteins in neuronal development and function will be described later.

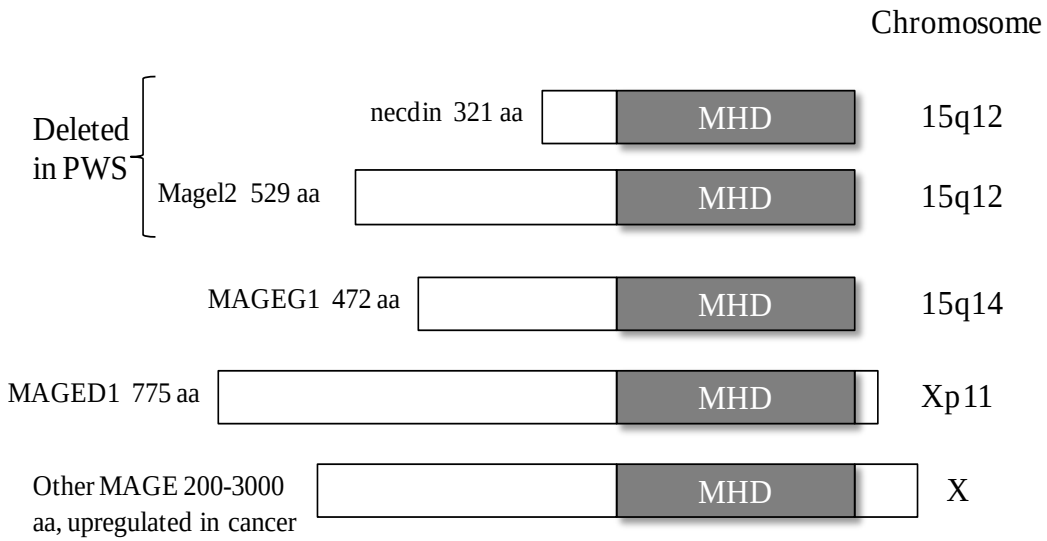

Figure 1-2. MAGE family of proteins.

Necdin and MAGEL2 are members of the melanoma antigen (MAGE) family of proteins, which all share a protein-protein interaction domain called the MAGE Homology Domain (MHD). MAGE proteins are involved in developmental processes or are up-regulated in cancer.

The remaining PWS protein-coding genes also have diverse cellular roles. The *ZNF127* gene encodes for Makorin3 (MKRN3), a protein that contains a RING (C3HC4) zinc finger motif (Jong et al. 1999). *C15ORF2* is expressed only from the paternal allele in fetal brain indicating it may play a role in the PWS phenotype but little else is currently known about its function (Buiting et al. 2007). *PWRN1* and *PWRN2* are highly expressed in testis and were identified as non-coding RNAs with multiple copies of partial duplications within the PWS/AS region. *SNURF-SNRPN* encodes two polypeptides: the SmN splicing factor, which is involved in RNA processing, and the SNRPN upstream reading frame (SNURF) polypeptide (Gray et al. 1999). It also encodes a long alternatively spliced transcript containing multiple copies of various snoRNA genes and extends downstream to partially overlap the *UBE3A* gene in the antisense orientation (Runte et al. 2001). Both the SNURF and SNRPN proteins are localized to the nucleus and are highly expressed in the brain (McAllister et al. 1988). *IPW* is located within the *SNURF-SNRPN* locus, is spliced and polyadenylated but only encodes a short polypeptide (Wevrick et al. 1994; Wevrick et al. 1996). The authors speculated that *IPW* may function as an RNA molecule and play a role in the imprinting process in the PWS/AS region.

SNORD116/PWCR1/HBII-85 was identified and characterized as an intronless gene in both humans and mice that does not code for a protein (de los Santos et al. 2000). A human-mouse conserved region (HMCR) in *SNORD116* was determined to have features of a C/D box snoRNA. The abundant transcripts are located in the nucleolus where these snoRNAs serve as methylation guidance RNAs in the modification of ribosomal and other small nuclear RNAs. *In vitro* studies have identified a role for *SNORD115/HBII-52* in the regulation of editing and alternative splicing of the serotonin 2C receptor (5HT2CR) mRNA (Kishore and Stamm 2006). Further studies of *SNORD115* have identified shorter versions of this transcript as being the predominant *SNORD115* RNA lost in PWS (Kishore et al. 2010). These novel *Snord115* transcripts, lacking the actual snoRNA stem, interact with heterogeneous nuclear ribonucleoproteins (hnRNPs), and not with proteins associated with canonical C/D box snoRNAs.

Defining a critical region for PWS

Studies in PWS individuals with balanced translocations have narrowed the PWS critical region, excluding *SNRPN-SNURF* as a major candidate (Schulze et al. 1996; Sun et al. 1996; Conroy et al. 1997; Kuslich et al. 1999; Wirth et al. 2001; Gallagher et al. 2002; Schule et al. 2005). Additionally, a report of one patient with a paternal deletion of *SNORD116* has identified that the loss of expression of this C/D box snoRNA cluster is a major contributor to the PWS phenotype (Sahoo et al. 2008). This patient had all seven major criteria but was in the 95% percentile for height. Two other patients with major features of PWS, including failure to thrive, hypogonadism, and excessive hyperphagia, with a deletion of *SNORD116* have also been described (de Smith et al. 2009; Duker et al. 2010). Another patient with atypical PWS characteristics possesses a translocation causing the loss of *MKRN3*, *NDN*, and *MAGEL2* while expression of *SNORD116* and *SNRPN* remains intact (Kanber et al. 2009). This patient is obese without obvious hyperphagia, has pain insensitivity, and developmental delay. Exclusion of *SNORD115* as a major contributor to the PWS phenotype was established in an AS family in which individuals who had a paternal loss of all copies of SNORD115 exhibited no obvious phenotype (Runte et al. 2005).

1.1.3 Multiple brain abnormalities are found in PWS individuals but major findings are likely caused by hypothalamic dysfunction

Features of PWS, including hypogonadism, growth hormone deficiency, hyperphagia, sleep disorders, and central adrenal insufficiency, indicate dysfunction or a developmental deficit of the hypothalamus. The portion of PWS individuals who are afflicted with a deficit of any of the hypothalamic-pituitary axes varies but it remains apparent that there is consistent involvement of the hypothalamus in many aspects of the PWS phenotype (Table 1-3). No structural lesions or abnormalities in the hypothalamus have been observed to date in PWS individuals. However, other intracranial abnormalities observed in MRI images of PWS patients include ventriculomegaly, sylvian fissure polymicrogyria, reduced brain volume in the parieto-occipital lobe, and incomplete insular closure

(Miller et al. 2007; Miller et al. 2007). Various pituitary abnormalities have been identified in PWS patients including a reduced or absent posterior pituitary bright spot (Miller et al. 1996; Iughetti et al. 2008), hypoplastic or globular pituitary glands (Miller et al. 2008), and a reduction in pituitary height in 50% of PWS patients (Iughetti et al. 2008).

Table 1-3. Percentage of PWS individuals with deficits in specific hypothalamic-pituitary axes.

Hypothalamic-pituitary axis	Clinical disorder	Percentage of PWS individuals	Reference
Adrenal	Central adrenal insufficiency	59%	A,B
Thyroid	Hypothyroidism	19-24%	C-G
Growth Hormone	Growth hormone deficiency	40-100%	H-L
Gonadal	Hypogonadism and delayed/absent puberty	67-100% (males) 56-76% (females)	M-P

A. (de Lind van Wijngaarden et al. 2008)
B. (Rudd et al. 1969)
C. (Butler et al. 2007)
D. (Festen et al. 2007)
E. (Miller et al. 2008)
F. (Diene et al. 2010)
G. (Vaiani et al. 2010)
H. (Burman et al. 2001)
I. (Beccaria et al. 1996)
J. (Beccaria et al. 1996)
K. (Grugni et al. 2006)
L. (Grugni et al. 2009)
M. (Greenswag 1987)
N. (Butler 1990)
O. (Crino et al. 2003)
P. (Eiholzer et al. 2006)

Further evidence for a hypothalamic deficit in PWS was demonstrated by a reduction in the number of oxytocin neurons in the paraventricular nucleus of the hypothalamus in post-mortem PWS brain samples (Swaab et al. 1995). The number of vasopressin neurons, a closely related population of neurons, was normal in the five PWS cases studied. Other studies of post-mortem brains from PWS individuals indicate no difference in levels of Neuropeotide Y (NPY) or Agouti-related peptide (AgRP), two orexigenic peptides in the hypothalamus, or GH-releasing hormone (GHRH) expressing cells (Goldstone et al. 2002; Goldstone et al. 2003). Recently, functional MRI studies have identified delayed activation of the hypothalamus after glucose ingestion in a small group of PWS patients when compared to weight-matched controls (Shapira et al. 2005). Further studies have identified increases in neural circuitry output in the prefrontal cortex of PWS individuals in response to food stimulation (Miller et al. 2007). There is also greater post-meal activation in food motivation network regions in the brain of PWS individuals when shown food pictures, which is opposite to that observed in healthy-weight controls (Holsen et al. 2006). PWS patients also show an increased response to both low- and high-calorie meals in the hypothalamus and orbitofrontal cortex, indicating altered neural circuitry in hunger and motivational pathways respectively (Dimitropoulos and Schultz 2008).

1.1.4 Autonomic dysfunction in PWS

Although autonomic dysfunction is not represented in the major criteria of PWS, it still encompasses a number of the neurological deficits observed in PWS individuals. Features of PWS caused by autonomic dysfunction include: reduced vomiting, abnormal thermoregulation, reduced saliva production, reduced gastrointestinal (GI) motility, respiratory depression, and decreased cardiac reflex activity (Wharton and Bresnan 1989; DiMario et al. 1994; DiMario et al. 1996; Hart 1998; Goldstone 2004; Choe et al. 2005). Altered parasympathetic tone is thought to be the cause of autonomic deficits in PWS. GI dysmotility has been linked with sudden death in PWS due to gastric rupture and necrosis, which is exacerbated by the decreased ability to sense pain (Schrander-Stumpel et al. 2004;

Stevenson et al. 2007). Respiratory depression, including multiple bouts of decreased oxygen saturation and excessive sedation, is observed in PWS children during a clonidine provocation test to evaluate growth hormone secretion (Hollman et al. 2010). Altered respiration could be explained by either central or peripheral autonomic dysfunction but is likely due to abnormalities in both systems. The autonomic nervous system (ANS) is centrally controlled by the hypothalamus and the brainstem. Integration defects in the central nervous system and neuronal deficits in the peripheral nervous system may combine to cause autonomic dysfunction in PWS.

1.1.5 Typical obesity versus obesity in PWS

Adipose tissue is an endocrine organ that secretes multiple hormones or adipokines including leptin and adiponectin, which are linked with obesity and insulin resistance (Antuna-Puente et al. 2008). Leptin acts on receptors in the arcuate nucleus of the hypothalamus to regulate food intake and energy expenditure. Plasma leptin levels correlate with the amount of fat mass in animals, with leptin resistance being a key component in the development of obesity. Mice homozygous for the deletion of leptin known as *ob/ob* or *obese* mice, exhibit hyperphagia leading to obesity, are glucose intolerant, have reduced fertility, are hypometabolic and have abnormal thermoregulation (Naggert et al. 1997). Leptin receptor knockout mice (*db/db* or *diabetes* mice) have a similar phenotype to that of the *ob/ob* mice. Adiponectin acts primarily through receptors in muscle and the liver (Yamauchi and Kadowaki 2008) but also acts in the hypothalamus to stimulate food intake and decrease energy expenditure in the fasted state (Kubota et al. 2007).

In typical obesity, circulating levels of adipokines are either increased (leptin) or decreased (adiponectin), and insulin resistance is common (Saltiel 2001; Qi et al. 2004; Fenton et al. 2009). Lower adiponectin levels, particularly the high molecular weight (HMW) form, correlate with insulin resistance in obese controls (Hu et al. 1996; Yamauchi and Kadowaki 2008). In PWS, serum leptin levels correlate with the degree of adiposity, however adiponectin levels are

increased compared to obese controls but are equivalent or slightly reduced compared to lean controls (Hoybye et al. 2004; Pagano et al. 2005; Kennedy et al. 2006; Festen et al. 2007; Haqq et al. 2007). Consistent with higher adiponectin levels, obese adults with PWS are relatively insulin sensitive compared to weight-matched controls (Bray et al. 1983; Talebizadeh and Butler 2005).

Ghrelin is secreted by the stomach and is an appetite-stimulating agent in its acylated form (Asakawa et al. 2001). Increased weight gain and adiposity are observed in rodents who are chronically administered ghrelin (Wren et al. 2001) and in humans, both lean and obese, ghrelin increases food intake and appetite (Wren et al. 2001; Druce et al. 2005). Ghrelin stimulates GH secretion by increasing production of GHRH in the arcuate nucleus of the hypothalamus. Fasting ghrelin levels are increased in children and adults with PWS when compared to weight-matched controls (Cummings et al. 2002; Haqq et al. 2003; Haqq et al. 2007; Feigerlova et al. 2008; Haqq et al. 2008; Prodam et al. 2009). Transgenic hyperghrelinemic mice are hyperphagic, glucose intolerant and leptin insensitive (Bewick et al. 2009), which is partially similar to what is seen in PWS individuals. Ghrelin knockout mice are not reduced in size nor do they exhibit changes in appetite under normal conditions (Sun et al. 2003), however under severe calorie restriction, ghrelin is required to induce GH secretion and preserve blood glucose levels (Zhao et al. 2010). Although, the PWS adipokine profile differs from that of typical obese individuals, it is similar to that observed in other GH-deficient disorders, including individuals with Laron syndrome, who are GH insensitive (Kanety et al. 2009).

1.1.6 PWS is a contiguous gene disorder

The vast majority of individuals with PWS lack expression of all paternally expressed genes on chromosome 15q11-q13, although recently, three patients have been described with deletions of the *SNORD116* snoRNA cluster and multiple major diagnostic criteria including hypogonadism, infantile hypotonia, and hyperphagia (Sahoo et al. 2008; de Smith et al. 2009; Duker et al. 2010). Studies in mice and a patient with atypical PWS (Kanber et al. 2009)

indicate a role for necdin and Magel2 in certain aspects of the PWS phenotype, including obesity, pain insensitivity, respiration deficits, growth hormone deficiency, and mental deficiency. Complex features of PWS including, global developmental delay and behavioural aspects, specifically obsessive compulsive disorder and addictive behaviours, are likely caused by the loss of multiple candidate genes as these characteristics are hypothesized to be caused by various gene-environment interactions in the general population.

1.1.7 Mouse models of PWS

As most PWS individuals lack expression of all paternally-expressed genes on chromosome 15q11-q13, it was necessary to develop a system that allows evaluation of the function of individual genes in the disease phenotype. Conveniently, the murine homologs of PWS genes are located in a syntenic region on mouse chromosome 7C and are also maternally imprinted. Targeted mutation or deletion of all or single PWS candidate genes in mice has aided researchers in determining the contribution of each individual gene to the disease phenotype and also in establishing their role during normal development in the mouse. The various PWS mouse models are summarized in Table 1-4 and are described in detail below to relate features of PWS with the loss of expression of genes in the entire PWS or of each individual candidate gene. Although mouse models are important in understanding the role of various genes in a disease phenotype, inherent differences between humans and mice sometimes make the interpretation of results difficult, especially with respect to complex traits like developmental delay and hyperphagia. However, the similarities between characteristics observed in individuals with PWS and in PWS mouse models support a role for the use of transgenic mice in the ongoing study of this complicated genetic disorder.

Table 1-4. Mouse models of Prader-Willi syndrome.

Mouse model	Phenotype	Reference
Multigene		
Uniparental disomy	100% postnatal lethality, reduced suckling	(Cattanach et al. 1992)
Transgene insertion	100% postnatal lethality, failure to thrive, feeding difficulties, reduced movement, irregular respiration, potential pancreatic defect	(Gabriel et al. 1999; Stefan et al. 2005)
Imprinting center (IC) deletion	75-100% postnatal lethality, failure to thrive, runted survivors	(Yang et al. 1998; Chamberlain et al. 2004)
Snrpn-Ube3a deletion	80% postnatal lethality, hypotonia, growth retardation	(Tsai et al. 1999)
Single gene		
Snord115/Mbii-52	No phenotype	(Ding et al. 2005)
Snord116/Mbii-85	0-15% postnatal lethality, postnatal growth retardation leading to runted adult size, increased anxiety, deficient motor learning, altered food intake and metabolism	(Ding et al. 2008)
Snrpn	No phenotype	(Yang et al. 1998)
Ndn	No phenotype in one strain (Tsai), 40-95% postnatal lethality (other three models) abnormal respiratory regulation, reduced muscle differentiation and regeneration, abnormal cellular migration, increased cell death and reduced axonal outgrowth in central and peripheral nervous systems in other three strains	(Gerard et al. 1999; Tsai et al. 1999; Muscatelli et al. 2000; Ren et al. 2003; Kuwako et al. 2005; Lee et al. 2005; Pagliardini et al. 2005; Deponti et al. 2007; Tennese et al. 2008; Bush and Wevrick 2010)
Magel2	Abnormal circadian rhythm, increased adiposity, reduced lean mass, reproductive decline, postnatal growth retardation, increased anxiety in novel settings, altered stress responses, partial growth hormone deficiency, hypothyroidism	(Bischof et al. 2007; Kozlov et al. 2007; Mercer et al. 2009; Mercer and Wevrick 2009; Tennese and Wevrick 2010)

Uniparental disomy and PWS region deletion mice

The first candidate PWS mouse model was created to evaluate the effect of imprinting in the PWS/AS critical region (Cattanach et al. 1992). The *Is1CtX*-autosome mouse has the central portion of mouse chromosome 7C, containing the PWS critical region, translocated to the X chromosome. Females carrying an unbalanced translocation with the portion of chromosome 7C translocated to the X chromosome and with two additional copies of chromosome 7C were mated to males carrying a balanced translocation. Only male progeny possessing two maternal copies of the PWS critical region were examined and exhibited postnatal lethality and reduced suckling, which is consistent with the failure to thrive and feeding difficulties observed in PWS neonates. Since the generation of this first mouse model, over a half-dozen transgenic mouse models involving the inactivation of genes in the PWS region have been produced.

A deletion model was created by a transgene insertion into the murine syntenic region which eliminated the complete PWS/AS homologous region (Gabriel et al. 1999). Postnatal lethality was the main feature observed in mice that paternally inherited the insertion due to extreme failure to thrive. Pups with the paternally-inherited transgene insertion also showed reduced growth, abnormal respiratory patterns, and reduced movement. Expression studies indicated that the transgene insertion caused the loss of expression of *Mkrn3, Ndn, Snurf-Snrpn,* and *Ipw*, all PWS candidate genes identified at the time of this study with *Magel2* being identified later. Further study of metabolic parameters in surviving transgene-deletion neonates identified deficits in insulin-like growth factor (Igf) system components in the liver as the cause of growth retardation (Stefan et al. 2005). Consistent with growth retardation, the transgene-deletion pups had reduced insulin and glucagon levels, and increased fat oxidation. The lethality in these transgene-deletion pups is due to severe hypoglycemia that results in compensatory increases in ghrelin levels, which occurs in an energy-deficient state (Zhao et al. 2010).

Another deletion mouse model removed the region between *Snurf-Snrpn* and *Ube3a* (Tsai et al. 1999). Pups inheriting the deletion paternally exhibited partial postnatal lethality, hypotonia, and growth deficiency. Imprinting status of necdin was normal in these mice indicating that the deletion does not affect the function of the imprinting center. This also indicated the presence of a gene between *Snrpn* and *Ipw* that was responsible for the postnatal lethality or failure to thrive observed in PWS neonates. This gene has subsequently been identified as the snoRNA C/D box gene cluster, *SNORD116/Snord116*.

IC deletion mouse models

Mice with a targeted deletion of the murine imprinting center (IC) have also been developed. The first IC deletion model exhibited postnatal lethality, reduced size of pups, failure to thrive, and mild hypotonia, which is similar to what is observed in models where the entire homologous region is mutated or deleted (Yang et al. 1998). A second IC deletion mouse model also exhibited postnatal lethality but the penetrance was strain-dependent (Chamberlain et al. 2004). IC-deletion survivors do not become obese and are fertile but remain runted throughout their life.

Ndn-null mouse models

Various single gene mutation transgenic mouse models have been created to elucidate the contribution of each individual gene to the PWS phenotype. Four independently generated mouse models in which necdin expression is lost exist. One mouse model has a targeted deletion of the complete open-reading frame of necdin with a beta-galactosidase reporter and a neomycin cassette, exhibits no obvious phenotype (Tsai et al. 1999). A second *Ndn*-null mouse model was created by replacing the promoter region and two-thirds of the necdin open-reading frame with a neomycin cassette (Muscatelli et al. 2000). These *Ndn*-null mice exhibit partial postnatal lethality, increased skin picking activity and improved spatial learning and memory, which are behaviours characteristic of PWS. Reduced numbers of gonadotropin-releasing hormone (GnRH) and

oxytocin-expressing neurons are also observed in this strain of *Ndn*-null mice. A third *Ndn*-null mouse model was created by insertion of a lacZ transgene into the necdin open-reading frame with subsequent excision of the neomycin cassette (Gerard et al. 1999). These mice exhibit almost 100% neonatal lethality on a C57Bl/6J background, while lethality is reduced to 85% in the F1 generation of a C57Bl/6J X CD1 cross. Therefore the study of adult mice in this third *Ndn*-null mouse model has not been as extensively performed as in the remaining models. The postnatal lethality observed in *Ndn*-null mice is due to deficits in the central respiratory rhythm generating center and altered modulation of serotonin in the medulla (Ren et al. 2003; Zanella et al. 2008). Abnormalities of axonal outgrowth in serotonergic and noradrenergic neuronal subtypes were observed in the brain of *Ndn*-null embryos (Lee et al. 2005). Commissural defects were also identified and defective pathfinding of internal capsule axons produced a whorl pattern adjacent to the hypothalamus in *Ndn*-null embryonic brains. Aberrant neuronal migration, defasciculation of axon tracts, and abnormal projections of axons, were observed in various nuclei in the medulla of *Ndn*-null embryos (Pagliardini et al. 2005). The fourth *Ndn*-null mouse model was produced in an ICR strain, and exhibited decreased pain sensitivity in response to heat, which was due to increased apoptosis in sensory DRG neurons and decreased substance-P containing neurons (Kuwako et al. 2005). The authors also identified an interaction between necdin and tropomysin-related kinase A (TrkA), leading to reduced activation of TrkA signaling in response to nerve growth factor (NGF) in *Ndn*-null neurons. A similar deficit in DRG development was described in one of the other previously described *Ndn*-null mouse models including increased apoptosis in the developing DRG of *Ndn*-null embryos (Andrieu et al. 2006). Defects in differentiation of GABAergic forebrain and hypothalamic GnRH neurons were also identified in two separate *Ndn*-null mouse models, due to loss of an interaction with *Dlx* and *Msx* transcription factors respectively (Kuwajima et al. 2006; Miller et al. 2009). *Ndn*-null mice have accelerated recovery of the hematopoietic system after myelosuppressive injury with increased levels of proliferating hematopoietic stem cells, indicating a role for necdin in negative

feedback (Kubota et al. 2009). Deficits in migration of various neuronal populations and muscle precursor cells are also observed in *Ndn*-null mice and will be discussed in depth later in this thesis.

Magel2-null mouse model

A *Magel2*-null mouse model was created by replacing the open-reading frame of *Magel2* with a lacZ transgene (Kozlov et al. 2007). This unique mouse model was made by our laboratory and is currently the only one described in the literature. *Magel2*-null mice are generally healthy, although they have deficits in behaviour and endocrine function. *Magel2*-null mice entrain appropriately to a normal light-dark cycle but exhibit an abnormal circadian rhythm of activity when placed in a completely dark environment (Kozlov et al. 2007). Specifically, their overall activity level is reduced, but they do show an increase in the number of bouts of activity. *Magel2*-null mice have reduced orexin levels and a decreased number of orexin-expressing neurons in the lateral hypothalamus, implicating Magel2 in the development of sleep disorders in PWS individuals. Postnatal growth retardation is observed in *Magel2*-null pups but by 5-6 weeks of age, their weights are similar to those of control littermates (Bischof et al. 2007). Increased adiposity and reduced muscle mass were identified by dual energy X-ray absorptiometry (DEXA) scan. They also exhibit increased anxiety in novel environments and have altered levels of serotonin and its metabolites (Mercer et al. 2009). *Magel2*-null mice have reproductive defects reminiscent of those observed in PWS including: reduced fertility with extended breeding intervals and early reproductive decline and termination; extended and irregular estrous cycles in females; and decreased testosterone in males (Mercer and Wevrick 2009). Further deficits in hypothalamic-pituitary axes in *Magel2*-null mice will be discussed in detail later in this thesis.

SnoRNA deletion mouse models

Two independent groups have mutated *Snord116* (*Pwcr1/MbII-85*) in mice and have subsequently studied the potential role of this gene in the PWS

phenotype. The first group noted postnatal lethality and growth retardation in their *Snord116*-null mice (Skryabin et al. 2007). The second group identified the same postnatal lethality and growth retardation, but also examined metabolic, endocrine, and behavioural parameters similar to those disrupted in PWS individuals (Ding et al. 2008). These *Snord116*-null mice are better able to maintain their weight on a low-calorie diet implying a more efficient metabolism, have altered fuel usage, increased ghrelin levels, increased anxiety, and have deficient motor learning.

A large radiation-induced deletion including the *Snord115* snoRNA C/D box gene cluster produced no phenotype in mice that paternally inherited the deletion (Ding et al. 2005). Another group identified an increase in editing but normal splicing of serotonin 2c receptor pre-RNA in surviving IC-deletion mice on a mixed strain (Doe et al. 2009). They also identified differences in behaviours regulated by serotonin 2c receptor pathways, including increased impulsivity and locomotor response when a food reward is present. These results indicate that loss of *Snord115* alone does not produce a phenotype but requires the loss of other genes in the PWS region to produce a significant effect.

1.2 Development of the autonomic nervous system (ANS)

Many of the clinical findings in PWS reflect dysfunction of the autonomic nervous system (ANS), including reduced salivation, gastrointestinal dysmotility, respiratory depression, decreased cardiac reflexes, reduced vomiting, and abnormal thermoregulation (Wharton and Bresnan 1989; DiMario et al. 1994; DiMario et al. 1996; Hart 1998; Goldstone 2004; Choe et al. 2005; Stevenson et al. 2007). The ANS is divided into three parts: sympathetic, parasympathetic, and enteric (Figure 1-3). As the ANS controls smooth and cardiac muscles, and exocrine glands, it is referred to as the visceral motor system. Classically, the sympathetic and parasympathetic nervous systems are described as having opposing effects on the regulation of physiological processes with the sympathetic division being dominant during stressful situations, while the parasympathetic

division is more prominent during times of relaxation (Dodd 1991). However both the sympathetic and parasympathetic nervous systems are tonically active and function along with the somatic motor system to regulate normal behavior and maintain homeostasis. The most classic example of opposing effects of the sympathetic and parasympathetic nervous systems is in the regulation of the cardiovascular system. Increases in heart rate and blood pressure induced by the sympathetic nervous system via epinephrine cause the activation of the vagal motor nucleus inducing a subsequent decrease in cardiac output and contractility by release of acetylcholine to return the heart rate back to baseline. Activation of both the sympathetic and parasympathetic nervous systems can occur simultaneously in the salivary glands and synergistically increase secretions and output of viscous and watery saliva respectively. The neurons of the enteric nervous system (ENS) populate the length of the gastrointestinal (GI) tract, the gall bladder, and pancreas, and are responsible for controlling secretions, motility, and vessel tone. Based on a limited number of studies of autonomic deficits in PWS, the likely cause of the deficits is diminished parasympathetic activity (DiMario et al. 1994; DiMario et al. 1996; Goldstone 2004). Autonomic dysfunction in PWS may be caused by a developmental defect in the peripheral or central nervous systems as multiple PWS candidate genes are expressed throughout embryogenesis (Lee et al. 2003).

Neural crest cells are a transient and migratory cell population required for the development of the peripheral nervous system, facial skeleton, and melanocytes. The generation of neural crest cells is dependent on the embryonal ectoderm, with their migration beginning after detachment from the dorsal neural tube epithelium. Members of the transformation growth factor beta (TGF-β) family induce specification of the dorsal neural tube and the generation of neural crest cells (Liem et al. 1997). The neural crest cells differentiate into neurons and begin to extend their axons into target tissues in response to guidance cues in their environment. Neurons that extend their axons into the right target tissue receive target-derived trophic factors that allow them to survive while those that fail to

extend their axons into the correct target tissue and do not receive an adequate supply of neurotrophic factors undergo programmed cell death.

Neurotrophins, glial cell-derived neurotrophic factor (GDNF) family members, and their cognate receptors are key regulators in the development and survival of autonomic neurons (Young et al. 2004). Neurotrophins bind to specific high-affinity receptors called tropomyosin-related kinases (Trk). The neurotrophins and their cognate receptors include: NGF and TrkA; brain-derived neurotrophic factor (BDNF) or neurotrophin (NT)-4/5 and TrkB; and NT-3 and TrkC. There is some level of cross-reactivity between the neurotrophins and Trk receptors with the presence of $p75^{NTR}$, the low affinity neurotrophin receptor, increasing the specificity of binding (Huang and Reichardt 2001). Ret (rearranged during transfection) is a tyrosine kinase and a membrane spanning receptor required for activation of downstream cellular pathways of GDNF family members (Airaksinen and Saarma 2002). GDNF family members with roles in the development of the ANS include: GDNF, neurturin, and artemin. The development of the different divisions of the ANS will be discussed below with respect to the requirement of neurotrophins and GDNF family members, although other guidance molecules and transcription factors are also required (Young et al. 2004).

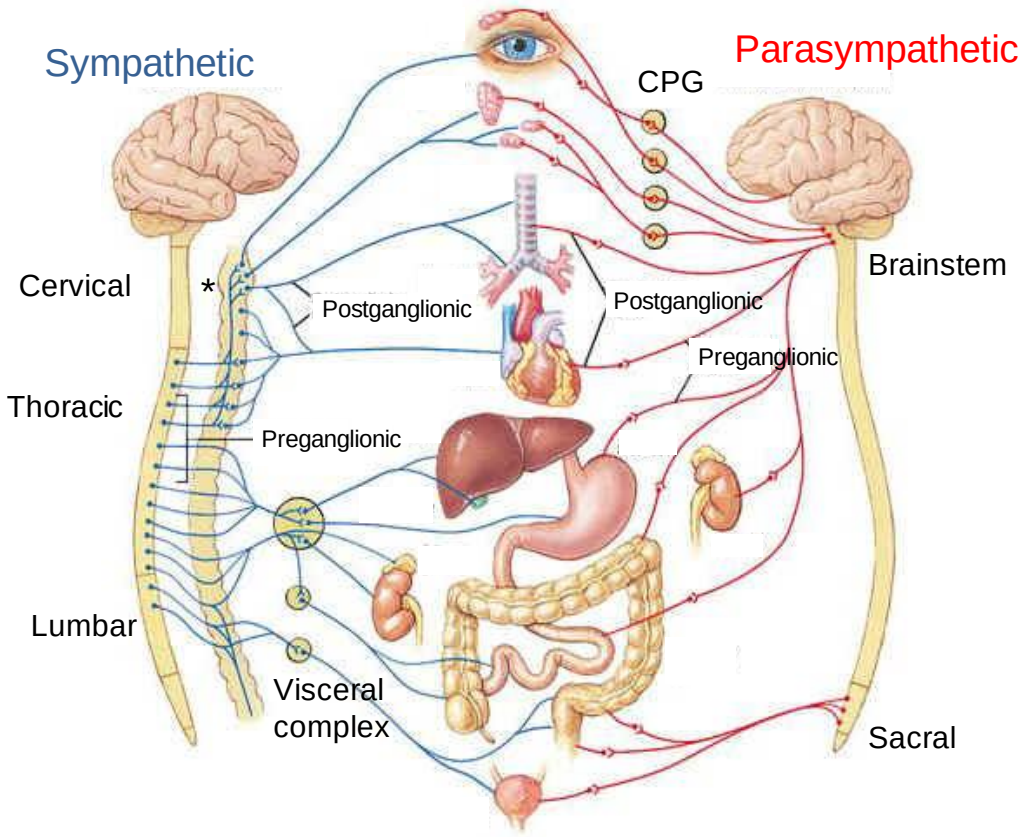

Figure 1-3. Schematic representation of the sympathetic and parasympathetic divisions of the autonomic nervous system.

The sympathetic nervous system is formed by preganglionic axons that project from the thoracic and lumbar regions of the spinal cord to ganglia adjacent to the spinal cord. The superior cervical ganglion (SCG) is marked with an asterisk and the visceral complex contains from rostral to caudal: the celiac, superior mesenteric, and inferior mesenteric ganglia. The parasympathetic preganglionic axons originate in cranial nerve nuclei in the brainstem, or in the sacral region of the spinal cord and project to ganglia on or within the tissues they innervate. Postganglionic axons in both the sympathetic and parasympathetic nervous systems are responsible for innervating tissues and organs. CPG, cranial parasympathetic ganglia.

(Adapted from http://www.mhhe.com/socscience/intro/ibank/ibank/0111.jpg)

1.2.1 Sympathetic nervous system

The postganglionic sympathetic ganglia are formed by the ventral migration of neural crest cells from the dorsal region of the developing neural tube to the mesenchyme lateral to the developing aorta. Bone morphogenetic proteins (BMPs) including BMP 2, 4, and 7, all members of the TGF-β family, are secreted by cells in the vicinity of the aorta to attract the migrating neural crest cells and induce differentiation of sympathetic precursors (Reissmann et al. 1996; Shah et al. 1996). Mash1 and Phox2b are transcription factors required for determining neuronal fate and whose expression first appears during sympathetic neuron differentiation. Expression of these genes leads to production of enzymes required for catecholamine biosynthesis including tyrosine hydroxylase (TH) and dopamine β-hydroxylase. The superior cervical ganglia (SCG) precursors must also migrate rostrally to their final location, the upper regions of the cervical vertebrae (Rubin 1985; Nishino et al. 1999). Other sympathetic precursors also migrate away from the developing sympathetic chain to populate the adrenal medulla with chromaffin cells that secrete epinephrine, and to form ganglia of the visceral complex in the mesentery (Le Douarin 1986).

Artemin is expressed by blood vessels along the migration path of SCG precursor cells and is required for migration of SCG neurons, as well as their survival (Honma et al. 2002). Consequently, loss of *GDNF family receptor alpha-3* (*Gfrα-3)* or *Ret* expression in transgenic mouse models leads to defects in SCG formation as artemin signals through these co-receptors (Nishino et al. 1999; Enomoto et al. 2001). Although there are significant defects in the SCG of *Gfrα-3* knockout mice due to the failure of rostral migration of SCG precursors, the remaining sympathetic chain ganglia appear to develop normally (Nishino et al. 1999). SCG target tissues are not adequately innervated in *Gfrα-3* knockout mice, which leads to increased cell death of SCG neurons as they do not receive the required amount of trophic factor. Survival of sympathetic neurons requires intact NT-3 and NGF signaling through TrkA, as *TrkA*-/- mice have increased cell death in the SCG perinatally and *TrkC*-/- mice do not exhibit any deficit in the SCG

(Huang and Reichardt 2001). Neurite outgrowth and entry of axons into target tissues by SCG neurons is dependent on various neurotrophins, including artemin, NGF, and NT-3, as determined by studies in transgenic mouse models (Honma et al. 2002; Glebova and Ginty 2004; Kuruvilla et al. 2004).

1.2.2 Parasympathetic nervous system

The development of postganglionic parasympathetic ganglia differs slightly from that of sympathetic ganglia as these neural crest cells must migrate further away from where they originate since their final location is adjacent to or within the tissue they innervate (Figure 1-3). Studies in transgenic mouse models have determined that the development of the cranial, cardiac, and sacral parasympathetic ganglia is dependent upon *Ret, Gdnf, neurturin, Gfrα*-1 and -2 expression to induce proliferation and migration of precursors, promote parasympathetic nerve outgrowth, and promote survival of parasympathetic neurons (Heuckeroth et al. 1999; Enomoto et al. 2000; Hiltunen et al. 2000; Hashino et al. 2001). The variability of the requirement of these factors for the development of parasympathetic ganglia indicates some redundancies in the function of GDNF family members and their receptors, but also indicates that certain roles developed independently (Rossi et al. 2000).

The cranial parasympathetic ganglia (CPGs) are composed of postganglionic neurons that are located near their target tissue. There are four CPGs: the sphenopalatine, ciliary, otic, and submandibular ganglia, which innervate the lacrimal and nasal mucosa glands; pupillary and ciliary muscles of the eye; parotid gland; and submandibular and sublingual glands; respectively (Dodd 1991). Parasympathetic innervation of organs and tissues in the cervical, thoracic, and lumbar regions mainly occurs through presynaptic ganglia that originate in the dorsal vagal nucleus and nucleus ambiguus in the brainstem and exit via the vagus nerve, cranial nerve (CN) X. Presynaptic ganglia of both the sympathetic and parasympathetic nervous systems are located within the lateral horn of the corresponding spinal cord segments.

1.2.3 Enteric nervous system (ENS)

The migration of vagal, rostral trunk, and sacral neural crest cells are responsible for populating the gut with enteric neurons. The neurons of the enteric nervous system (ENS) are arranged in interconnected plexuses, which are formed by ganglia and the nerve fibers connecting them. The two major intrinsic plexuses are the myenteric and submucosal plexuses, which are located between the circular and longitudinal muscles, and within the connective tissue of the submucosa respectively (Dodd 1991). Subsets of neurons within the ENS include sensory neurons that respond to mechanical distension and changes within the chemical environment; and interneurons and motor neurons that control the muscles of the gut wall, the vasculature, and secretions from the mucosa. The ENS innervates the gastrointestinal (GI) tract, pancreas, and gall bladder, and regulates GI vascular tone, motility, and endocrine and exocrine secretions (Goyal and Hirano 1996). Regulation of immune and inflammatory processes, such as viral or bacterial gastroenteritis and inflammatory bowel disease, also requires functional enteric neurons. The ENS can function autonomously however extrinsic innervation by the sympathetic and parasympathetic nervous systems and regulation via the central nervous system is required for its contribution to homeostasis.

Post-migratory enteric neurons express Phox2b, Ret, and $p75^{NTR}$, with labelled cells being present only in the stomach at embryonic day (E) 9.5-10 in mice (Young et al. 2000). The neural crest cells migrate caudally from the stomach along the GI tract, with the entire gut being colonized by E14. Gene inactivating mutations in *Ret* tyrosine kinase are a cause of Hirschsprung's disease, which is characterized by congenital megacolon due to aganglionosis of the intestines. *Ret, GDNF, and Gfrα-1* deficient mice die shortly after birth due to severe renal dysgenesis and lack of ENS neurons throughout the GI tract (Schuchardt et al. 1994; Moore et al. 1996; Pichel et al. 1996; Sanchez et al. 1996; Enomoto et al. 1998). *Ntn*-/- and *Gfrα*-2-/- mice have less severe defects in the

ENS, including reduced density of myenteric plexus innervations and reduced GI motility (Heuckeroth et al. 1999; Rossi et al. 1999).

The ANS is responsible for maintaining a homeostatic environment by regulating the tone of the sympathetic and parasympathetic nervous systems in organs and tissues throughout the body. Alterations in sympathetic and parasympathetic tone regulate changes in heart rate, blood pressure, thermoregulation, micturition, respiratory rate, and salivation. The enteric nervous system regulates the function of the gut and also innervates the pancreas and gall bladder. In conjunction with the central nervous system, the ENS controls endocrine and exocrine secretions, gut motility, and GI vessel tone. Features of PWS indicate disruption of the ANS, particularly altered tone of the parasympathetic nervous system, including thickened saliva, respiratory depression, and GI dysmotility. Investigation of the ANS in a mouse model of PWS will be presented in this thesis and provide further support for necdin in the development of the peripheral nervous system.

1.3 Hypothalamic regulation of homeostasis

Growth hormone deficiency, hypogonadism, central adrenal insufficiency, hypothyroidism, sleep disturbances, and hyperphagia are aspects of the PWS phenotype caused by hypothalamic deficits. To date, it is not known which of the PWS candidate genes contributes to hypothalamic development and function. Although various PWS candidate genes are expressed throughout the nervous system, necdin and Magel2 are the most highly expressed in the hypothalamus during development (Lee et al. 2003). This suggests that loss of these genes compromises the development of the hypothalamus and their absence may contribute to altered function of the hypothalamus and its output via the pituitary gland and the autonomic nervous system. Evaluation of various hypothalamic-pituitary-endocrine axes in a mouse model of PWS will be presented in this thesis.

1.3.1 Anatomy and structure of the neuroendocrine hypothalamus

The hypothalamus develops from the ventral portion of the diencephalon and can be identified as a distinct population of cells by E9.5 in the developing mouse brain. Sonic hedgehog plays an important role in patterning the vertebrate hypothalamus (Mathieu et al. 2002; Manning et al. 2006; Szabo et al. 2009). While sonic hedgehog functions early in the formation of the hypothalamus, BMPs act later, with this temporal difference in expression being required for region-specific transcriptional profiles (Szarek et al. 2010). Studies in mice and zebrafish have also determined that NODAL and members of the Wnt signaling pathway, respectively, are necessary for proper induction and patterning of the hypothalamus during neurodevelopment (Szarek et al. 2010).

The vertebrate hypothalamus is situated between the optic chiasm (anterior) and the mammillary body (posterior). It is also located dorsal to the pituitary gland and ventral to the thalamus (Figure 1-4). The hypothalamus can be divided into three medial to lateral areas: periventricular, medial, and lateral; and four rostral-caudal regions: preoptic, anterior, tuberal, and mammillary. The periventricular region contains four distinct nuclei: arcuate nucleus, paraventricular nucleus, suprachiasmatic nucleus, and periventricular nucleus; many of these nuclei are important in feeding regulation. The medial region contains the anterior hypothalamus, dorsomedial nucleus, and ventromedial nucleus. The lateral hypothalamic region contains the preoptic area and the hypothalamic area. The suprachiasmatic nucleus and supraoptic nucleus are located at the midline, directly above the optic chiasm.

Two different neurosecretory cell populations are located throughout the hypothalamus: parvocellular and magnocellular neurons. The parvocellular neurons can be sub-divided into groups according to the type of anterior pituitary hormone secretion that they stimulate or inhibit: gonadotropin-releasing hormone (GnRH), corticotrophin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), GH-releasing hormone (GHRH), dopamine, gonadotropin-inhibiting hormone, and somatostatin; and are located in the basal portion of the medial and

periventricular regions of the hypothalamus (Figure 1-4) (Szarek et al. 2010). Magnocellular neurons located in the paraventricular nucleus and supraoptic nucleus secrete either arginine-vasopressin (AVP) or oxytocin with their axons projecting directly to the posterior pituitary (Kupfermann 2001). In addition to regulating the function of the pituitary gland, projections from the hypothalamus also regulate the function of the autonomic nervous system with the most prominent responses involving the sympathetic nervous system.

1.3.2 Anatomy of the pituitary gland

The pituitary gland or hypophysis is located below the hypothalamus and rests in a depression of the sphenoid bone at the base of the skull called the sella turcica. There are three lobes in the mature pituitary gland that are formed by different embryonic tissues, including the anterior lobe or adenohypophysis, intermediate lobe or pars intermedia, and posterior lobe or neurohypophysis (Figure 1-4). The anterior and intermediate lobes are formed by the rostral migration of oral ectoderm called Rathke's pouch from the embryonic palate, toward the developing diencephalon, while the posterior lobe develops from a projection of the neural ectoderm.

The anterior lobe secretes hormones from five different specialized cell types in response to activation from the corresponding hypothalamic hormone. The hormones of the anterior pituitary include: growth hormone (GH) secreted by somatotropes, adrenocorticotropin hormone (ACTH) secreted by corticotropes, prolactin secreted by lactotropes, follicle-stimulating and luteinizing hormones (FSH and LH) secreted by gonadotropes, and thyroid-stimulating hormone (TSH) secreted by thyrotropes. The intermediate lobe produces melanocyte stimulating hormone (MSH), though this is often included as a product of the anterior lobe. The posterior lobe contains projections of AVP and oxytocin neurons that originate in the paraventricular nucleus and supraoptic nucleus with their release being controlled by the hypothalamus. AVP and CRH positively coordinate release of ACTH while somatostatin inhibits both TSH and GH secretion, with

their release being dependent on TRH and GHRH production increasing to levels that override this inhibition. Prolactin is secreted when dopamine levels are adequately reduced to remove its inhibitory effects on lactotropes. Coordination of gonadotropin releasing and inhibiting hormones determines the temporal regulation and amount of FSH and LH secretion.

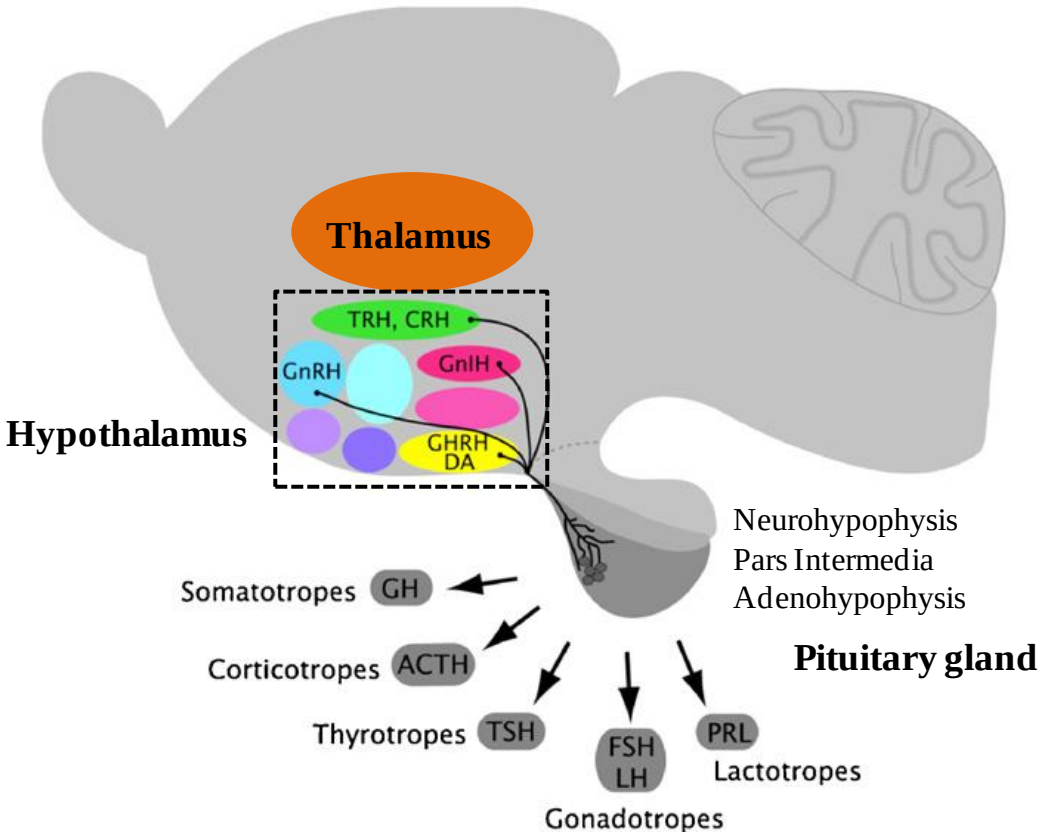

Figure 1-4. Organization of the hypothalamic nuclei.

A sagittal view of the hypothalamus and pituitary gland. The hypothalamus is organized into distinct regions of neuronal cell bodies or nuclei. The represented nuclei are, starting from the top, moving clockwise, and ending in the centre: paraventricular nucleus (green); dorsomedial nucleus (dark pink); ventromedial nucleus (light pink); arcuate nucleus (yellow); supraoptic nucleus (blue); suprachiasmatic nucleus (purple); preoptic area (light blue); and anterior hypothalamus (aqua). Also depicted are the neuroendocrine hypophysiotropic factors and their neuronal projections through the median eminence to the type of cells they stimulate in the anterior pituitary. CRH, corticotrophin-releasing hormone; TRH, thyrotropin-releasing hormone; GnIH, gonadotropin-inhibiting hormone; GHRH, growth hormone releasing hormone; DA, dopamine; GnRH, gonadotropin-releasing hormone; GH, growth hormone; ACTH, adrenocorticopin hormone; TSH, thyroid stimulating hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PRL, prolactin. [Adapted from (Szarek et al. 2010)]

1.3.3 Hypothalamic-autonomic output

The autonomic nervous system (ANS) is controlled at three different levels. Peripheral information enters the central nervous system via the spinal cord or brainstem nuclei, particularly the nucleus tractus solitaris located within the medulla. Brainstem and spinal neuron groups control the reflex actions of the ANS by receiving afferent information via parasympathetic cranial nerves and sympathetic spinal nerves. Next, information is integrated by forebrain neurons and is transmitted to the hypothalamus, the most important site for integration of the neuroendocrine and autonomic systems (Saper 2008). Hypothalamic neurons integrate sensory, visceral, and limbic information from the hippocampus, cerebellum, basal ganglia, cortex, and reticular formation; and compare it to a homeostatic set point. Autonomic efferent responses originate in the lateral hypothalamus but must be integrated with information from the anterior and posterior pituitary, before inducing a systemic response (Dodd 1991). Although the hypothalamus is a major contributor to autonomic regulation, it is not absolutely required as transection of the brainstem above the pons does not affect respiratory or cardiovascular function. The nucleus tractus solitaris is the major regulating center of autonomic function in the brainstem (Figure 1-5) as it controls simple reflex circuits and coordinates systemic responses with higher and lower brain regions. Control of various physiological functions including swallowing, temperature, blood pressure, peristalsis, micturition, blood glucose levels, respiration, heart rate, and electrolyte balance is regulated by the ANS.

The hypothalamus and pituitary gland are responsible for regulating the secretion of hormones that target various endocrine organs and alter systemic functions. The ANS controls visceral functions and coordinates with the hypothalamus to regulate responses via the peripheral nervous system and the pituitary. Endocrine abnormalities and autonomic dysfunction are commonly observed in individuals with PWS. I examined the autonomic nervous system, and hypothalamic-pituitary-endocrine axes in mouse models of PWS to determine

if these features could be explained by loss of *NDN* and *MAGEL2*, two PWS candidate genes.

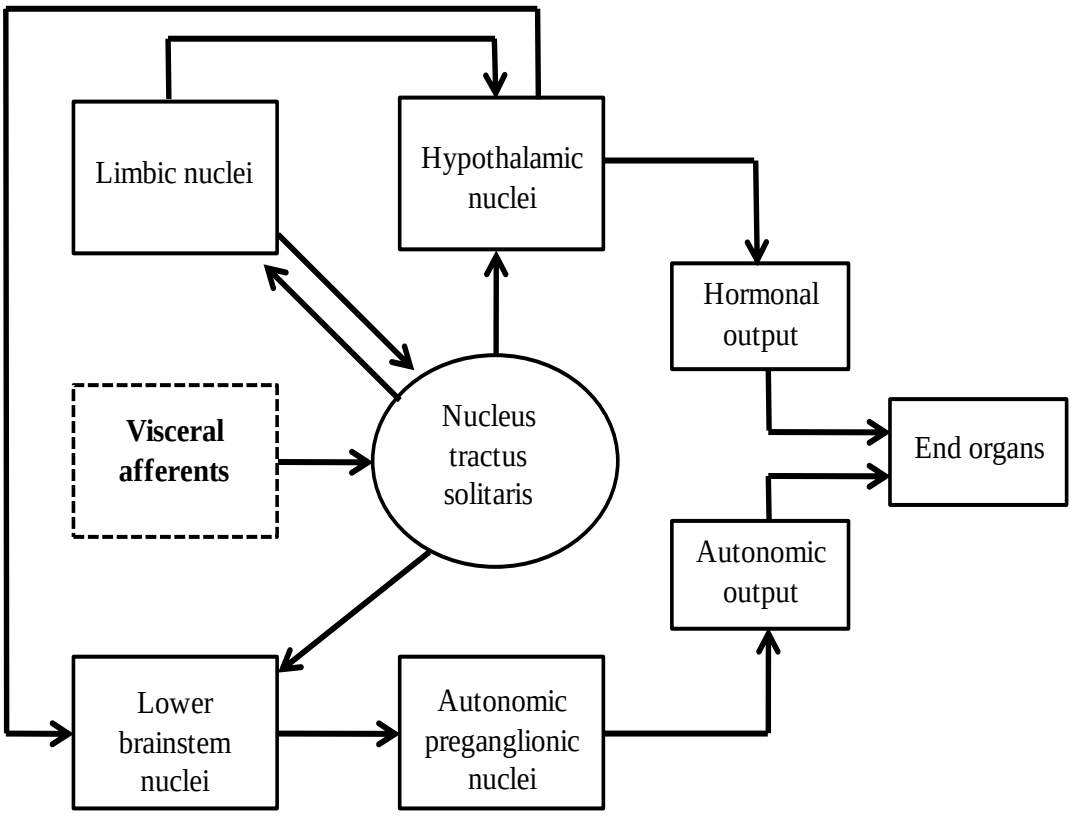

Figure 1-5. Regulation of autonomic output in the central nervous system.
The illustration depicts the integration of visceral afferent information in the central nervous system. The nucleus tractus solitaris is the major coordinating center in the brainstem and regulates simple reflexes and higher level integration with other portions of the brain. The hypothalamus directly controls hormonal release via the pituitary gland to influence autonomic function but also has connections with the brainstem to control output via autonomic efferents. [Adapted from (Dodd 1991)]

1.4 Function of the hypothalamic-pituitary-end organ axes

The hypothalamus receives input from higher cortical centres and the brainstem, and signals from the periphery including hormones, metabolites, and peptides in the blood (Kupfermann 2001). It integrates these complex signals and regulates feedback mechanisms through the neuroendocrine pituitary gland and the autonomic nervous system. The pituitary gland is often referred to as the master endocrine gland as it secretes hormones that regulate homeostasis, and trophic factors that stimulate peripheral endocrine organs. Upon signal integration, stimulating hormones from the hypothalamus bind to their cognate receptors in the pituitary and cause the secretion of their respective hormones from the anterior pituitary (Figure 1-6). The anterior pituitary hormones enter the hypophyseal portal system and travel through the bloodstream to reach their specific target organs. The hypothalamic-pituitary axis regulates its output via multiple positive and negative feedback loops. The increase in peripheral hormone feeds back to the hypothalamus and pituitary and inhibits further secretion of their stimulation hormones, which allows the return of peripheral hormone levels to baseline.

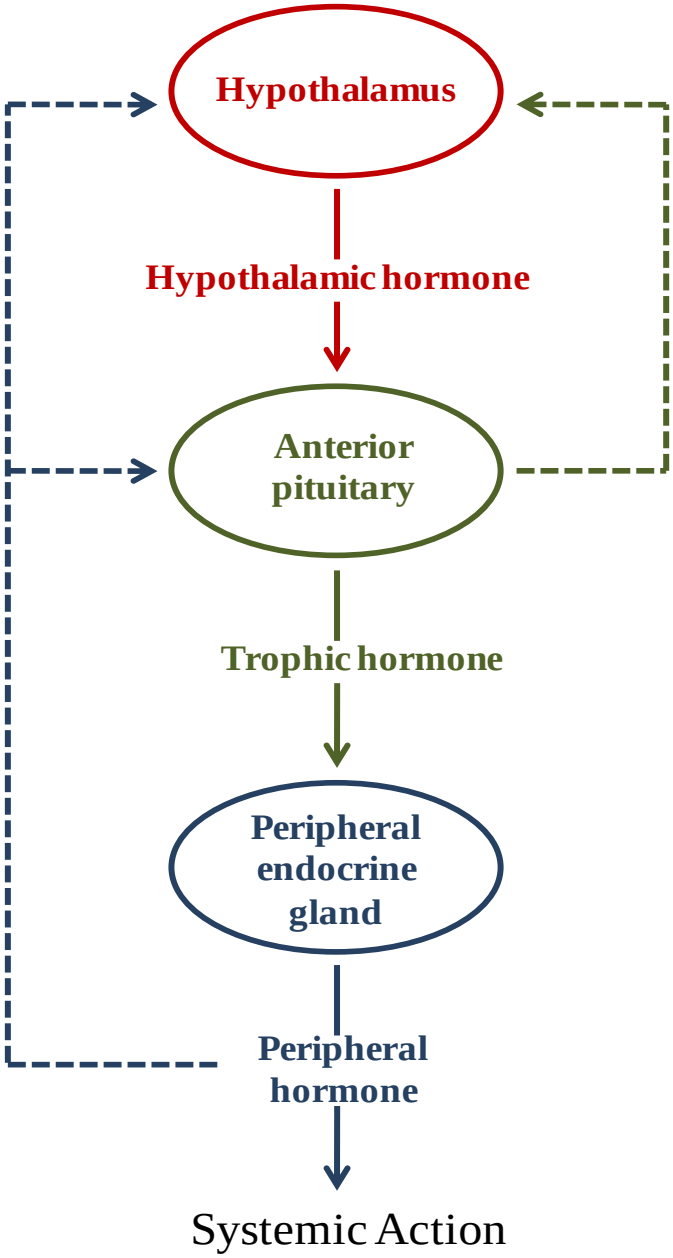

Figure 1-6. General representation of the hypothalamic-pituitary axis.

The hypothalamus secretes a hormone that stimulates the release of an anterior pituitary hormone. The trophic pituitary hormone enters systemic circulation and stimulates the release of a peripheral hormone at the target end organ. This peripheral hormone produces the desired physiologic response and also negatively feeds back to prevent further secretion of hypothalamic and pituitary hormones. (Positive feedback = solid line, negative feedback = dashed line.)

1.4.1 Hypothalamic-pituitary-gonadal axis

Briefly, the hypothalamus produces gonadotropin-releasing hormone (GnRH), which causes the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Guber 2007). In females, pulses of FSH and LH coordinate the menstrual cycle and induce secretion of estrogen which is responsible for breast development and other secondary sexual female characteristics. In males, FSH acts on germ cells to stimulate the production of sperm, while LH stimulates the production and secretion of testosterone from interstitial cells. The hypothalamic-pituitary-gonadal axis is dysregulated in PWS (Table 1-3). The deficits observed include cryptorchidism or undescended testes in males; and hypogonadism and delayed or absent puberty involving diminished secondary sexual characteristics in both sexes (Greenswag 1987; Butler 1990; Crino et al. 2003; Eiholzer et al. 2006). The cause of hypogonadism has both peripheral and central components, which include high FSH and a primary defect in germ cell maturation; and low LH, respectively. This axis has previously been examined in our *Magel2*-null mice with features reminiscent of those observed in PWS (see above section 1.1.7) (Mercer and Wevrick 2009).

1.4.2 Hypothalamic-pituitary-adrenal (HPA) axis

The adrenal cortex secretes glucocorticoids, cortisol in humans and corticosterone in mice, in response to ACTH, which is released in a circadian pattern but whose secretion can also be induced by systemic stress and hypoglycemia. Cortisol has multiple systemic roles and is involved in protein catabolism, fat synthesis and redistribution, gluconeogenesis and reducing inflammation (Guber 2007). ACTH release by corticotrope cells in the anterior pituitary is stimulated by CRH release from the lateral part of the medial paraventricular nucleus and also by AVP release from the posterior pituitary (Figure 1-7). The HPA axis consists of various feedback loops that control cortisol synthesis and secretion. When cortisol levels increase, CRH, AVP, and ACTH release is suppressed, which returns cortisol levels back to baseline. Conversely, when cortisol levels reach their nadir at the end of the presumptive

day, ACTH production is increased, thereby raising cortisol levels. This tightly regulated feedback loop allows levels of ACTH and cortisol to remain in a narrow range allowing even slight changes in levels of either hormone to alter the other.

Stressors affecting the HPA axis in rodents can be divided into two broad categories: psychogenic including restraint and shaker stress; and physiologic including starvation, fluid deprivation, and hypoglycemia. Restraint produces primarily an anticipatory rather than a physiological stress, activating the HPA axis through input from peripheral sensory information, the nucleus tractus solitaris in the brainstem, and forebrain reactive responses, with the various signals being integrated in the paraventricular nucleus (Herman et al. 2003; Ulrich-Lai and Herman 2009). Insulin-induced hypoglycemia has a direct effect on glucose-sensing neurons in the ventromedial nucleus, eliciting a counter-regulatory survival response by inducing glucagon and corticosterone secretion (Routh 2003).

Various tests can be administered to evaluate the integrity of the HPA axis. As serum cortisol levels fluctuate throughout the day, stimulation and suppression tests are more common in the evaluation of the HPA axis. ACTH stimulation acts directly upon the adrenal gland and will identify individuals with primary adrenal insufficiency as cortisol secretion will be inadequate. The insulin tolerance test (ITT) is the gold standard to access the integrity of the entire HPA axis as hypoglycemia induces sequential CRH, ACTH and cortisol secretion. However the ITT is not recommended in PWS individuals due to the high probability of central adrenal insufficiency (de Lind van Wijngaarden et al. 2008). Metyrapone is an inhibitor of 11β-hydroxylase, the enzyme that converts 11-deoxycortisol to cortisol, and when administered causes an increase in both CRH and ACTH levels due to a drop in cortisol levels (Figure 1-7) (Guber 2007). In people who cannot undergo an ITT, metyrapone stimulation can be used to identify individuals with adrenal insufficiency. Dexamethasone suppression of the HPA axis decreases cortisol levels as it negatively feedbacks to the

hypothalamus and pituitary, preventing secretion of CRH and ACTH; and will identify individuals with hypercortisolism.

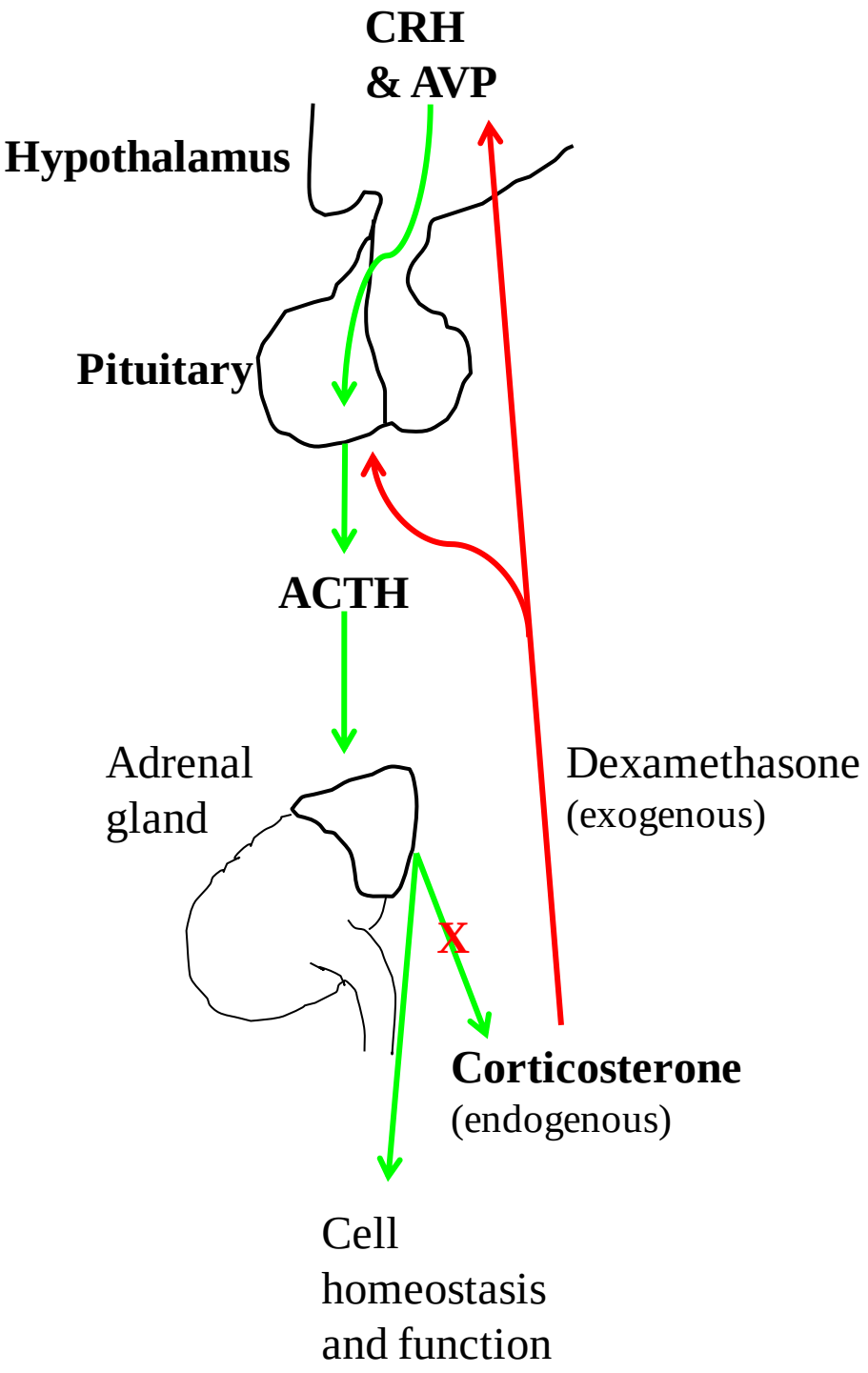

Figure 1-7. The hypothalamic-pituitary-adrenal (HPA) axis.

Stress signals integrated in the hypothalamus cause the release of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) which stimulates adrenocorticotropin hormone (ACTH) secretion from the anterior pituitary. ACTH stimulates production and secretion of corticosterone, a glucocorticoid that functions in cell homeostasis. Returning to a baseline physiological state requires corticosterone to negatively feedback to the hypothalamus and pituitary, suppressing further release of CRH and ACTH. Red lines indicate negative feedback or suppression, and green lines indicate positive feedback or stimulation. The red X denotes the location of action of metyrapone, an inhibitor of cortisol synthesis. Dexamethasone, a synthetic glucocorticoid, negatively affects CRH and ACTH secretion. (Adapted from Henry's Clinical Diagnosis and Management by Laboratory Methods, Chapter 24; (Guber 2007))

1.4.3 Hypothalamic-pituitary-growth hormone axis

Growth hormone (GH) is secreted by somatotropes in the anterior pituitary in response to GH-releasing hormone (GHRH) from the arcuate nucleus and is inhibited by somatostatin. Regulation of GH synthesis and secretion is complex with GHRH determining the amplitude of the GH pulses with intermittent somatostain withdrawal regulating the timing of the pulses (Figure 1-8). GH pulses differ in frequency and quantity between species. In rodents, GH pulses occur approximately every 90 minutes in females and every 3-4 hours in males, with differences between peaks and troughs being more pronounced in males (Jansson et al. 1985). In humans, maximal GH release occurs in the second half of the night and is mediated by circadian rhythm. GH binds to receptors in the liver which causes the synthesis and secretion of somatomedins or insulin-like growth factors (IGFs). IGFs are effectors of the GH axis that circulate in the blood bound to IGF-binding proteins (IGFBPs), with IGFBP-3 being the most important. GH directly acts on osteoblasts and chondrocytes to induce linear growth but also acts indirectly through IGF-1 by inducing organ and muscle growth and anabolic metabolism (Guber 2007) (Figure 1-8). GH is responsible for maintaining a normal body composition by increasing lean mass and reducing fat mass. GH deficiency in childhood causes short stature while excessive secretion of GH in childhood leads to gigantism or acromegaly in adulthood.

Ghrelin is a recent addition to the GH signaling pathway and is an acylated peptide secreted by the stomach prior to meal induction in response to hunger (Kojima et al. 1999). It binds to an orphan receptor, growth hormone secretagogue receptor (GHSR), in the hypothalamus and anterior pituitary gland and stimulates GH secretion in conjunction with GHRH (Figure 1-8). Due to the connection of ghrelin with the GH pathway, it was anticipated that ghrelin-null mice would exhibit phenotypes consistent with GH deficiency. However, ghrelin-null mice are not reduced in size, nor do they exhibit any changes in appetite (Sun et al. 2003). Ghrelin stimulation of GH secretion has been studied extensively in rodents and is absent in *Ghsr*-/- mice, and reduced in mouse models of obesity

(Asakawa et al. 2001; Wren et al. 2001; Sun et al. 2004; Iwakura et al. 2007; Sun et al. 2008). The gold-standard test to evaluate for GH deficiency is the insulin tolerance test (ITT) (Bray et al. 1983; Guber 2007). When the ITT cannot be administered, the next test of choice is GHRH stimulation with arginine or provocation with clonidine, but it is also not recommended in PWS as it exacerbates existing respiratory deficits (Hollman et al. 2010). Insulin and arginine stimulate GHRH production while clonidine inhibits somatostatin. Using GHRH alone stimulates the pituitary but bypasses the hypothalamus, consequently it can miss 50% of people with tertiary GH deficiency or hypothalamic dysfunction.

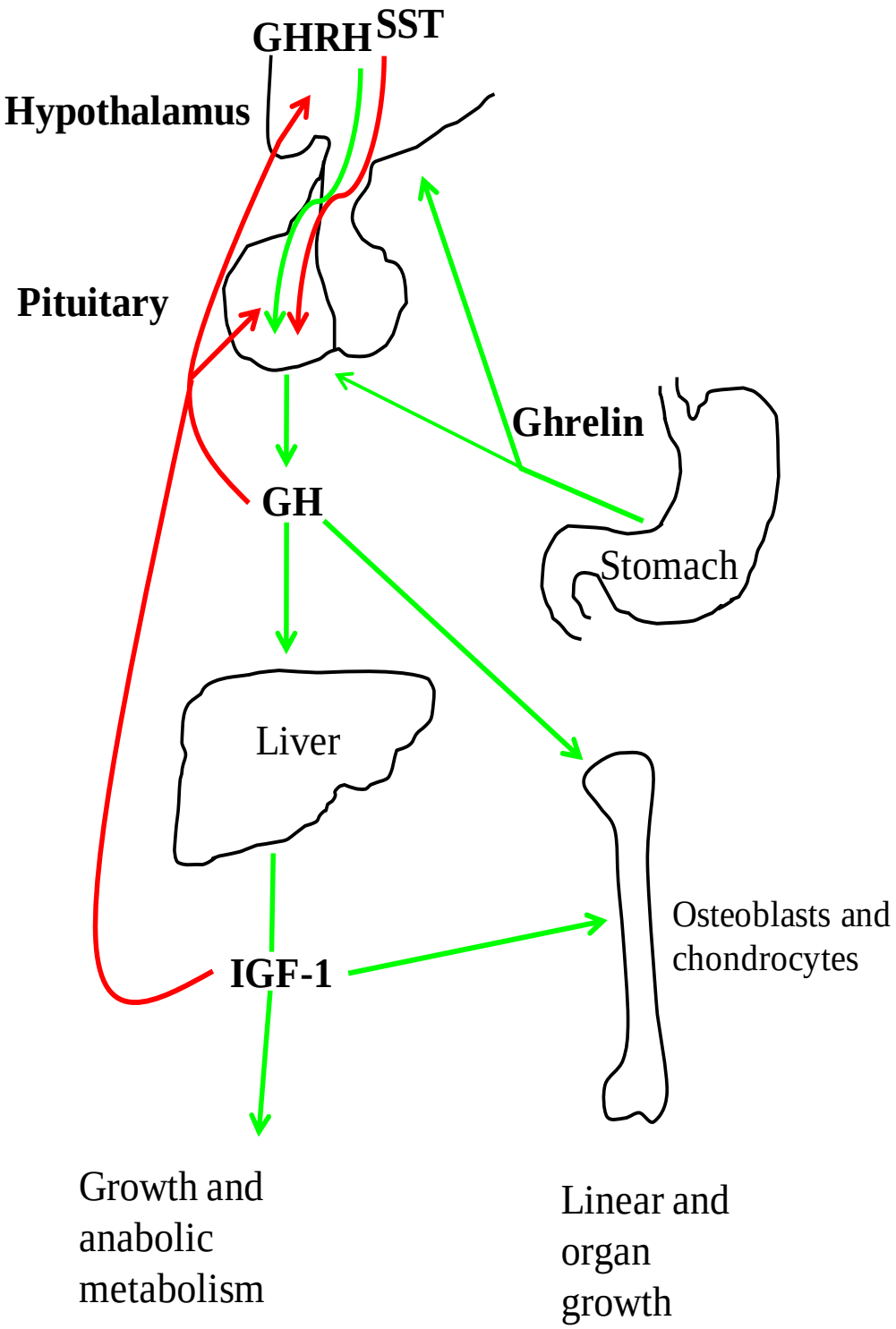
GHRH
SST
Hypothalamus
Pituitary
Ghrelin
GH
Stomach
Liver
IGF-1
Osteoblasts and
chondrocytes
Growth and
anabolic
metabolism
Linear and
organ
growth

Figure 1-8. Hypothalamic-pituitary-growth hormone axis.

The secretion of growth hormone-releasing hormone (GHRH) by the hypothalamus is pulsatile due to the cyclical removal of inhibition by reducing somatostatin (SST) expression. GHRH stimulates growth hormone (GH) secretion from the anterior pituitary which has direct and indirect effects on growth and metabolism via bone tissue and the liver respectively. Insulin-like growth factor (IGF-1) is produced by the liver to execute systemic functions. GH and IGF-1 negatively feed back to prevent further secretion of GHRH. Ghrelin is secreted by the stomach in low energy states and induces the production of GHRH, thus stimulating GH secretion. Red lines indicate negative feedback or suppression, and green lines indicate positive feedback or stimulation. (Adapted from Henry's Clinical Diagnosis and Management by Laboratory Methods, Chapter 24; (Guber 2007))

1.4.4 Hypothalamic-pituitary-thyroid axis

The thyroid gland is divided into lobules that are composed of follicles, which are ring-like structures with the centre containing colloid, thyroid hormone, and thyroglobulin. Follicles are the site of synthesis and storage of thyroid hormone. Synthesis of thyroid hormone requires a ready supply of iodine and an intact hypothalamic-pituitary-thyroid axis. The hypothalamus releases thyrotropin-releasing hormone (TRH) which stimulates secretion of thyroid-stimulating hormone (TSH) from thyrotropes in the anterior pituitary (Figure 1-9). TSH stimulates thyroid hormone synthesis by inducing iodine transport into follicular cells, where thyroid hormones, thyroxine (T_4) and 3,5,3'-triiodothyronine (T_3), are produced on a thyroglobulin backbone. The metabolic steps required for synthesis of thyroid hormone are complex [Reviewed in (Guber 2007)]. All circulating T_4 is produced in the thyroid gland while the majority of circulating T_3 is produced by deiodination of T_4 in other peripheral tissues. T_3 is the more active form produced by the thyroid but free T_4 tends to be measured in diagnostic studies. Thyroid hormones are required for normal development due to their roles in regulating protein synthesis and controlling metabolism.

Primary hypothyroidism is caused by a defect within the thyroid gland that causes a reduction in the levels of circulating thyroid hormones, subsequently leading to an increase in TSH secretion. Central hypothyroidism indicates a defect in the production or secretion of TSH. When iodide is abundant, T_4 is the dominant product and when TSH secretion is increased, T_3 release is enhanced (Guber 2007). Obese individuals often have increased TSH levels and normal to elevated T_3 levels, which is the opposite to that observed in anorexic individuals (Reinehr et al. 2008). TSH is the most sensitive test to evaluate the integrity of the hypothalamic-pituitary-thyroid axis but only if the axis is intact. Most individuals with hypothyroidism will have high TSH levels but if the pituitary or hypothalamus is malfunctioning, T_3 and T_4 levels may be within the normal range.

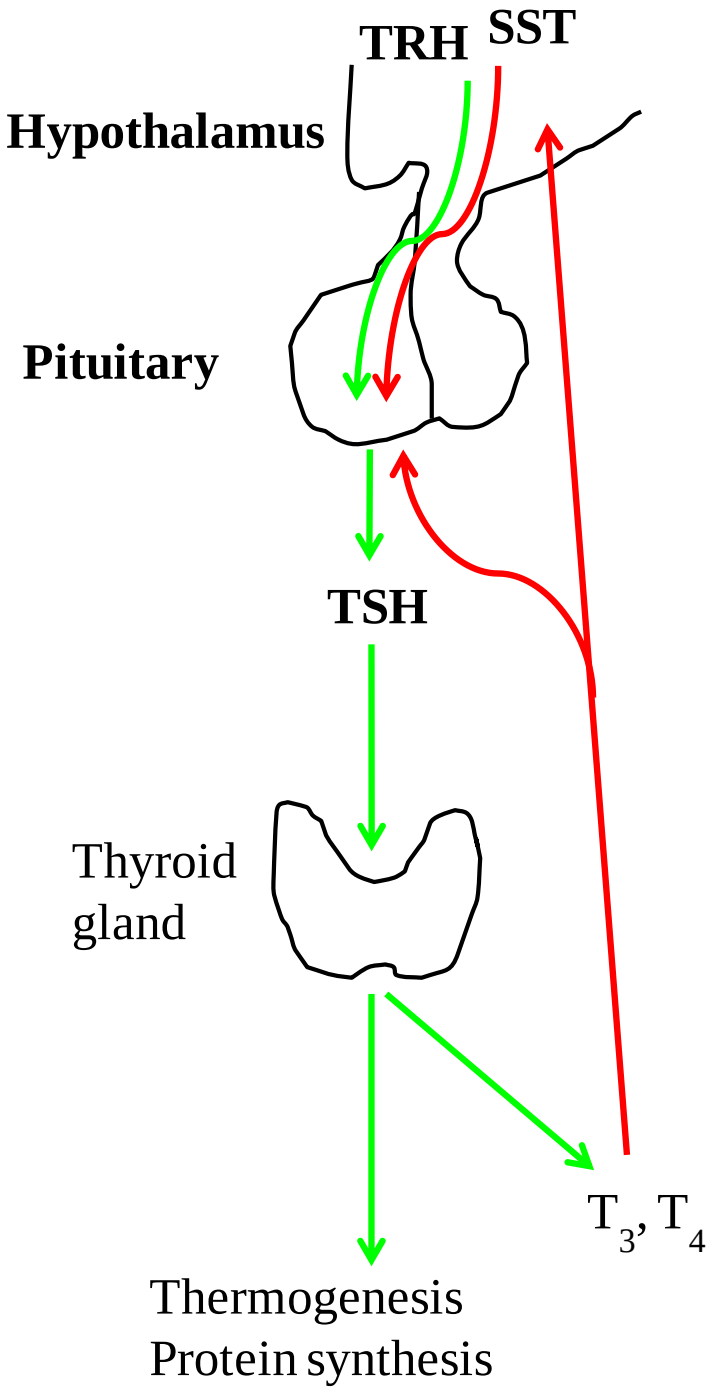
TRH
SST
Hypothalamus
Pituitary
TSH
Thyroid gland
T_3, T_4
Thermogenesis
Protein synthesis

Figure 1-9. Hypothalamic-pituitary-thyroid axis.

Thyrotropin-releasing hormone (TRH) is secreted by the hypothalamus and stimulates the

secretion of thyroid-stimulating hormone (TSH), when inhibition by somatostatin (SST) has been relieved. TSH induces the production of thyroid hormones, T_3 and T_4, which act systemically to modulate thermogenesis and protein synthesis for proper development. Thyroid hormones negatively feed back to the hypothalamus and pituitary and prevent further secretion of TRH and TSH. Red lines indicate negative feedback or suppression, and green lines indicate positive feedback or stimulation. [Adapted from Henry's Clinical Diagnosis and Management by Laboratory Methods, Chapter 24; (Guber 2007)]

1.5 Hypothesis and Summary of Studies

When I joined the Wevrick laboratory in early 2004, we were examining defects in the brain of *Ndn*-null embryos and had also identified reduced axonal outgrowth, abnormal axonal bundling, and reduced survival of cultured *Ndn*-null sympathetic neurons (Lee et al. 2005). Previous studies had identified a role for necdin in terminal differentiation of sensory neurons and cells transfected with necdin (Kobayashi et al. 2002; Takazaki et al. 2002). Reduced numbers of certain neuronal populations were also identified in the hypothalamus of another *Ndn*-null mouse model (Muscatelli et al. 2000). Since loss of necdin affects the development of various neuronal subtypes, I hypothesized that necdin is essential for the development of the sympathetic nervous system and potentially the entire peripheral autonomic nervous system (ANS), as many clinical aspects of the PWS phenotype suggest dysfunction of the ANS. The work presented in this thesis identifies a role for necdin in the development of the sympathetic nervous system, a novel role for necdin in cellular migration, and confirms a role for necdin in axonal outgrowth (Tennese et al. 2008).

PWS is a neurodevelopmental disorder, specifically thought to be caused by dysfunction of the hypothalamus. Phenotypes such as reduced fertility and blunted circadian rhythm, observed in *Magel2*-null mice indicate a role for this protein in the homeostatic function of the hypothalamus (Kozlov et al. 2007; Mercer and Wevrick 2009). At the time I became interested in the hypothalamic-pituitary axes in PWS, no hypothalamic function studies in other PWS mouse models existed. Therefore, as both necdin and Magel2 are highly expressed in the hypothalamus, I hypothesized that loss of *Magel2* or *Ndn* in mice may cause deficits in hypothalamic-pituitary axes similar to those observed in PWS. Further work documented in this thesis identifies growth hormone deficiency, altered stress responses, a delayed counter-regulatory response to hypoglycemia, and hypothyroidism in *Magel2*-null mice (Tennese and Wevrick 2010). The data I present on necdin and Magel2 support a role for these MAGE proteins in the neurological phenotype of PWS, although further studies will be required to

understand specific hypothalamic deficits in both transgenic mouse models with respect to output via the autonomic nervous system and pituitary gland.

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Chapter 2. Loss of the Prader-Willi syndrome protein necdin causes defective outgrowth, migration, and survival of sympathetic neurons.

Parts of this chapter have been previously published.

Tennese, A.A., Gee, C.B., and Wevrick, R. (2008) Loss of the Prader-Willi syndrome protein necdin causes defective outgrowth, migration and survival of embryonic sympathetic neurons. *Developmental Dynamics.* 237(7): 1935-43.

With the exception of the innervation data presented in Figure 2-4, Alysa Tennese completed all of the experiments presented in this chapter. C.B. Gee was a summer student who I supervised in the collection of data in the experiments in Figure 2-4 B-E.

2.1 Introduction

Many clinically relevant aspects of Prader-Willi syndrome (PWS) suggest dysfunction of the autonomic nervous system (Wharton and Bresnan 1989; DiMario et al. 1994; DiMario et al. 1996; Hart 1998; Goldstone 2004; Stevenson et al. 2004; Choe et al. 2005). *NDN*, encoding the protein necdin, is one of several genes deleted in individuals with PWS, and is highly expressed throughout the nervous system, including peripheral autonomic neurons (Aizawa et al. 1992; MacDonald and Wevrick 1997). A role for necdin in terminal differentiation of neurons is supported by studies showing that PC12 cells transfected with necdin have increased differentiation and accelerated neurite outgrowth (Tcherpakov et al. 2002), that repression of necdin in embryonic dorsal root ganglia suppresses their differentiation (Takazaki et al. 2002), and that expression of necdin induces neurite outgrowth in neuroblastoma cells (Kobayashi et al. 2002). The development and survival of autonomic neurons is dependent on neurotrophin signaling pathways, as demonstrated by defects in transgenic mice harboring mutations in neurotrophic factors and their cognate receptors (Ernfors et al. 1994; Ernfors et al. 1994; Kuruvilla et al. 2004). Necdin participates in neurotrophin

signaling through its interaction with both the low affinity neurotrophin receptor $p75^{NTR}$ and tropomyosin-regulated kinase TrkA in multiple systems, including transiently transfected cells, P19 embryonal carcinoma cells, and dorsal root ganglia (Tcherpakov et al. 2002; Andrieu et al. 2003 ; Kuwako et al. 2005).

Two strains of mice with gene-targeted deletions of necdin exhibit a high degree of neonatal lethality (Gerard et al. 1999; Muscatelli et al. 2000) due to a defect in central respiratory rhythm generation (Ren et al. 2003). In the most severe cases, *Ndn*-null mice have commissural defects in the forebrain and axonal extension, bundling, and branching defects in central nervous system neurons (Lee et al. 2005). An increase in embryonic apoptosis was identified in embryonic sensory neurons of *Ndn*-null mice, accompanied by loss of sensory innervation in the adult mice (Takazaki et al. 2002; Kuwako et al. 2005; Andrieu et al. 2006). A reduced number of GABAergic but normal numbers of glutamatergic neurons were noted in the *Ndn*-null developing forebrain, suggesting that survival varies among neuronal populations with necdin-deficiency (Kuwajima et al. 2004). Further studies indicate that the reduced number of GABAergic interneurons in the neocortex of *Ndn*-null embryos is caused by a reduction in differentiation and migration of these neurons, as loss of necdin expression impairs the action of Dlx transcription factors (Kuwajima et al. 2006; Kuwajima et al. 2010). One additional strain of *Ndn*-null mice has no phenotype (Tsai et al. 1999).

We now demonstrate that necdin is essential for the normal development of the autonomic nervous system *in vivo*, by examining the embryonic development of the autonomic nervous system in *Ndn*-null mice. The neurons that form the superior cervical ganglia (SCG) are most profoundly affected by necdin-deficiency, displaying reduced axonal outgrowth, reduced innervation of the salivary glands, and increased cell death. Surprisingly, we found impaired rostral migration of a subset of SCG neurons, demonstrating a previously unknown function for necdin in development. Other sympathetic ganglia in the thoracic and lumbar regions appear to be formed normally, although a deficit in

axonal outgrowth is observed. The adrenal medulla, cranial parasympathetic ganglia, and enteric nervous system (ENS) are also unaffected by loss of necdin expression. These data extend the range of neuronal subtypes affected by necdin-deficiency to include the autonomic nervous system (ANS).

2.2 Materials and methods

Mouse Breeding and Genotyping

All animal studies procedures were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Health Sciences Animal Policy and Welfare Committee for the University of Alberta. The *Ndn* mice were originally on a mixed genetic background (W9.5 (129S1) / C57Bl/6) and subsequently back-crossed to C57Bl/6 for at least 14 generations. The *Ndn* mouse colony was maintained by breeding *Ndn*-/+ female mice carrying a maternally inherited *Ndn-lacZ* knockin allele with C57Bl/6 male mice to generate heterozygous, functionally wild-type offspring. C57Bl/6 female mice were then bred to *Ndn*-/+ male mice carrying a maternally inherited *Ndn-lacZ* knockin allele, to generate *Ndn*+/- embryos carrying a paternally inherited *lacZ* knockin allele (referred to as *Ndn*-null) and *Ndn*+/+ (control littermate) embryos. Because of imprinting that silences the maternally inherited allele, heterozygous *Ndn*-null mice with a paternally inherited *lacZ* knockin allele retain expression only of this mutant allele and have no expression of *Ndn*. Mice were genotyped from tissue samples or ear notch biopsies.

Tissue preparation

Tissues and whole embryos were placed in 4% paraformaldehyde (Sigma-Aldrich, Oakville, ON) in phosphate-buffered saline (PFA) for 1-2 days at 4°C, then were cryoprotected in 30% sucrose in phosphate-buffered saline (PBS), frozen in Cryomatrix Frozen Specimen Embedding Medium (Shandon ThermoScientific, Pittsburgh, PA) over dry ice and stored at -80°C. Frozen tissues were sectioned at 20-30µm on a Leica CM1900 cryostat (Leica Microsystems, Heidleberger Strasse, Germany) at a cutting temperature of -20°C

to -25°C. Sections were placed on Superfrost Plus charged slides (Fisher Scientific, Ottawa, ON) and allowed to air-dry at room temperature for 30 minutes before being stored at -80°C until processing.

For whole-mount tissue preparation for immunohistochemistry (IHC), embryos or adult tissues were placed in 4% PFA over 1-2 days at 4°C, then were dehydrated in a methanol series: 25%, 50%, 80% and 100%, and stored at -20°C until processing. For whole-mount tissue preparation for acetylcholinesterase staining, adult gastrointestinal tracts were placed in 4% PFA for 1-2 hours at RT, then were placed in supersaturated sodium sulfate solution (Sigma-Aldrich) at 4°C until processing.

Thionine staining

Slides were baked at 42°C for 2 hours on a slide warmer (Fisher Scientific). Slides were transferred to a coplin jar and dehydrated in 50% ethanol. Slides were then rinsed in distilled water and stained in thionine for 30 seconds. Slides were washed twice in distilled water, destained in 95% and 100% ethanol, dehydrated in xylene, mounted with Entellan mounting media (EM Industries, Gibbstown, NJ) and coverslipped.

Immunohistochemistry (IHC) on cryosections

Slides were warmed at room temperature (RT) for 15 minutes, outlined with ImmEdge Pen (Vector Labs, Burlington, ON), and placed in PBS with 0.1% Tween (PBST) in a coplin jar for 5 minutes. Blocking solution (10% horse or goat serum and 0.3% TritonX-100 in PBS) was placed on the slides for 1 hour at room temperature (RT) in a humidified chamber. Primary antibody (see Table 2-1) was diluted in blocking solution (1% horse or goat serum and 0.3% TritonX-100 in PBS), applied to slides, and incubated overnight at RT in a humidified chamber. Slides were washed three times for five minutes each in PBST.

Secondary antibody (see Table 2-1) was diluted in 1% horse or goat serum in PBST and incubated at RT for 60-90 minutes in a humidified chamber. Slides were washed three times for five minutes each in PBST, stained with Hoescht stain for 10 minutes, and washed a final time in PBST. Slides were mounted with 1:3 glycerol in PBS, coverslipped, and stored at -20°C until examined under a Leica DMRE microscope (Leica Microsystems). Images were captured with a SensiCam camera (Cooke Corporation, Auburn Hills, MI), and Northern Eclipse software (Media Cybernetics, Carlsbad, CA).

Whole-mount IHC

Embryos were incubated overnight in 20% DMSO/80% methanol with 3% hydrogen peroxide to quench endogenous peroxidase activity. Tissues were rehydrated, blocked overnight in blocking solution (5% skim milk powder/5% DMSO/1% Tween-20 in PBS) and incubated for 48-72 hrs at 4°C with a sheep anti-tyrosine hydroxylase (TH) antibody (Table 2-1) in blocking solution. Next, tissues were incubated overnight with a peroxidase-conjugated anti-sheep secondary antibody in blocking solution. After washing in PBS with 1% Triton X-100, tissues were incubated with diaminobenzidine for 30-60 minutes in the dark then the color was developed by adding hydrogen peroxide solution. The reaction was stopped by placing tissues in 4% PFA overnight. Upon dehydration in a methanol series, tissues were cleared in 1:2 benzyl alcohol: benzyl benzoate (Sigma-Aldrich) and stored at 4°C until images were captured on a Leica DMRE microscope (Leica Microsystems).

Antibodies

Table 2-1. List of antibodies used in this study.

Antibody name	Species	Dilution	Company and location
Choline acetyltransferase (ChAT)	Goat	1:400	Millipore/Chemicon, Temecula, CA
Tyrosine hydroxylase (TH)	Rabbit	1:2000	Millipore/Chemicon, Temecula, CA
Tyrosine hydroxylase (TH)	Sheep	1:500	Millipore/Chemicon,

			Temecula, CA
Neurofilament 2H3	Mouse	1:2000	DSHB, Iowa City, IA
Ki67	Rabbit	1:1000	Millipore/Chemicon, Temecula, CA
Cleaved caspase 3	Rabbit	1:1000	Cell Signaling, Danvers, MA
Neuron-specific βIII tubulin (TUJ1)	Mouse	1:1000	Millipore/Chemicon, Temecula, CA
anti-rabbit Alexa Fluor 488 or 594	Goat	1:1000	Molecular Probes/Invitrogen, Burlington, ON
anti-mouse Alexa Fluor 488 or 594	Goat	1:1000	Molecular Probes/Invitrogen, Burlington, ON
anti-goat Cy3	Donkey	1:200	Jackson Immunoresearch Laboratories Inc., Westgrove, PA

Whole-mount acetylcholinesterase staining

Tissue was incubated at RT for 2-4 hours in a staining solution containing 0.2 mM ethopropazine-HCl, 4 mM acetylthiocholine iodide, 10 mM glycine, 2 mM cupric sulfate and 65 mM sodium acetate (pH 5.5). The colour reaction was developed by incubation in a 1.25% solution of sodium sulfide (pH 6.0) for 90 seconds. Tissues were rinsed with copious quantities of water, immersed in a 1:1 solution of glycerol/PBST, and examined and photographed with a Leica MZ8 dissecting microscope (Leica Microsystems) and a Nikon digital camera. All chemicals were obtained from Sigma-Aldrich.

RNA in situ hybridization

The *Phox2b* probe was kindly provided by Dr. J.F. Brunet (CNRS, Marseilles). The *Ret* probe pmcRET7 was kindly provided by Dr. V. Pachnis (National Institute for Medical Research, London, UK).

Labelling of probes

Plasmid DNA was digested with *EcoRI* and *NotI* for the Phox2b and Ret constructs respectively, separated on a 1% agarose gel, and extraction purified from the gel with a QIAquick gel purification kit (Qiagen, Mississauga, ON) according to manufacturer's instructions. Antisense riboprobes were generated using T3 and T7 RNA polymerases for Phox2b and Ret respectively (Invitrogen, Burlington, ON), and a digoxygenin (DIG) RNA Labelling Mix (Roche Diagnostics, Mississauga, ON).

Hybridization and detection

Slides mounted with 20-30µm cryosections of mouse embryonic tissues were baked at 42°C for 2 hours. Slides were washed twice in diethyl pyrocarbonate (DEPC)-treated PBS for 5 minutes each, washed twice in 100mM glycine in DEPC-treated PBS for 5 minutes each, and were incubated in 0.3% Triton X-100 (Fisher Scientific) in DEPC-treated PBS for 15 minutes. Slides were washed twice in DEPC-treated PBS on an orbital shaker (VWR Scientific, Edmonton, AB) for 5 minutes each, then were incubated for 30 minutes at 37°C with 1X TE (100mM Tris-Cl, pH 8 and 50mM EDTA pH 8) with 1µg/ml proteinase K to permeabilize the tissue. To stop the proteinase K reaction, slides were post-fixed in 4% PFA made with DEPC-treated PBS at 4°C for 5 minutes. Slides were washed twice with DEPC-treated PBS for 5 minutes each. The hybridization steps were performed in a humidified chamber with 50% formamide used as the humidifying solution. Slides were covered in 200-250µl of hybridization solution containing 50% formamide, 5X saline-sodium citrate (SSC), 1% sodium dodecyl sulfate (SDS), 50µg/ml yeast RNA, and 50µg/ml heparin, and incubated at 65-68°C for 15-30 minutes. While the slides are incubating in the pre-hybridization solution, 35-65ng of probe was mixed in 200µl of hybridization solution, incubated at 80°C for 5-10 minutes, and vortexed immediately prior to use. Slides were covered in 200µl of hybridization solution with the desired probe and incubated O/N in the humidified chamber at 65-68°C. Slides were covered with pieces of Parafilm (Cole-Parmer Canada Inc., Montreal,

QC) to prevent evaporation of solution during both pre-hybridization and hybridization steps.

The following morning, slides were transferred to a coplin jar and rinsed on a rocker in 2X SSC, containing 3M NaCl and 300 mM sodium citrate for 20-30 minutes. Slides were washed twice in 2X SSC with 50% formamide and 1% SDS for 15 minutes each, then were washed twice in 1X SSC for 15 minutes each, and were washed twice in 0.1X SSC for 30 minutes each, with all steps proceeding at 65-68°C. Slides were washed twice for 10 minutes each in DIG detection buffer 1, containing 100mM Tris pH 7.5 and 150mM NaCl. Slides were returned to a humidified chamber with DIG detection buffer 1 as the humidifying agent and were incubated in blocking solution [DIG detection buffer 1 with 2% BMB (Roche Blocking agent reconstituted with maleic acid) and 0.1% Triton X-100] for 30 minutes at RT. Slides were incubated with 1:2000 anti-digoxygenin (DIG) antibody in blocking solution for 2 hours at RT. Antibody was detected with NBT/BCIP stock solution (Roche Diagnostics) in a solution of 100mM Tris pH 9.5, 100mM NaCl, and 50mM $MgCl_2$, with 200mM levamisole. Colour development was stopped in a solution of 10mM Tris pH 8, and 1mM EDTA. Slides were dehydrated and subsequently rehydrated in successive ethanol/PBS solutions to remove background staining. Slides were mounted with Aquamount (VWR Scientific) and coverslipped. Images were obtained using a Leica DMRE microscope.

Image analysis

All organs were reconstructed from images of 25-30µm serial sections with Adobe Photoshop and analyzed with ImageJ software. Relative SCG sizes were estimated by thionine staining as the area occupied on the three to five consecutive sections that contained the entire ganglion, in multiple embryos from multiple litters. SCG cell numbers were determined by counting nuclei labeled with Hoechst (total cells) or labeled by anti-cleaved caspase-3 (for cell death) or Ki67 (for proliferation). The innervation of SCG target organs was measured as the amount of anti-TH immunofluorescence as a fraction of total area occupied by

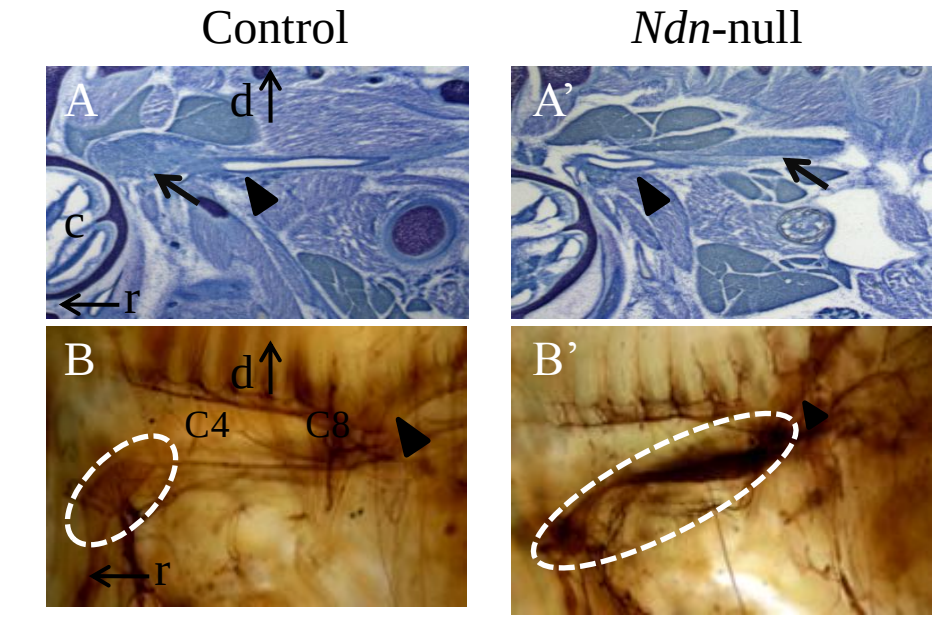
each organ. The relative size of the adrenal gland was estimated by the area labeled with the anti-TH antibody on sections through the entire gland, measuring every third section. Statistical comparisons between *Ndn*-null and control littermate embryos were made using the Student t-test with $p \leq 0.05$ considered significant.

2.3 Results

2.3.1 Superior Cervical Ganglia Are Smaller and Are Abnormally Located in the Ndn-null Embryo

The progenitors of sympathetic neurons are neural crest cells that migrate from the dorsal region of the developing neural tube toward the aorta to form the sympathetic trunk. One sub-lineage of these neural crest cells then migrates rostrally to the upper regions of the cervical vertebrae in a stereotypical manner to form the SCG (Nishino et al. 1999). Additional neural crest cells from the vagal region colonize the enteric nervous system (ENS) in the mid- and hindgut. SCG precursors can first be identified as a cell grouping at embryonic day (E) 10.5 in mice and by E11.5, they are aligned along the cervical vertebral column between cervical vertebra 1 (C1) and C7. At E12.5, SCG cells proliferate and begin to migrate away from the stellate ganglia, the closest caudally located ganglia in the sympathetic chain. By E14.5, the SCG neurons have reached their final

destination at the bifurcation of the carotid artery. To better understand the role of necdin in the development of the sympathetic nervous system, we examined the SCG using thionine staining for general morphology and tyrosine hydroxylase (TH) immunohistochemistry to detect sympathetic neurons. At both E16.5 and E18.5, we detected TH-positive neurons in the sympathetic chain ganglia of both *Ndn*-null and control littermate embryos, which suggests that sympathetic noradrenergic neurons are normally specified in the absence of necdin. The most rostral of these neurons form the SCG, which migrate to the bifurcation of the carotid artery between cervical vertebral levels C1-C4 (Figure 2-1A). While some SCG neurons have migrated to the C1-C4 region in *Ndn*-null embryos, the majority of SCG neurons are consistently located more caudally and are significantly reduced in number compared with control littermates (Figure 2-1 B,C). We also noted increased variability in the rostrocaudal location of the SCG between the left and right sides in *Ndn*-null embryos. These data suggest that, although noradrenergic specification proceeds normally, necdin is required for the final localization of the superior cervical ganglia.


Control
Ndn-null
A
d
c
r
A'
B
d
C4
C8
r
B'

C

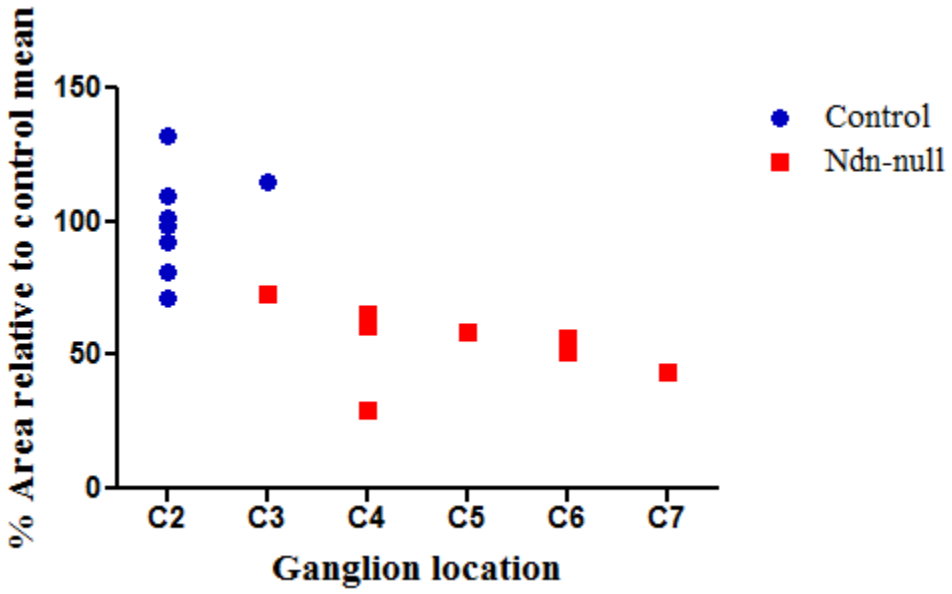
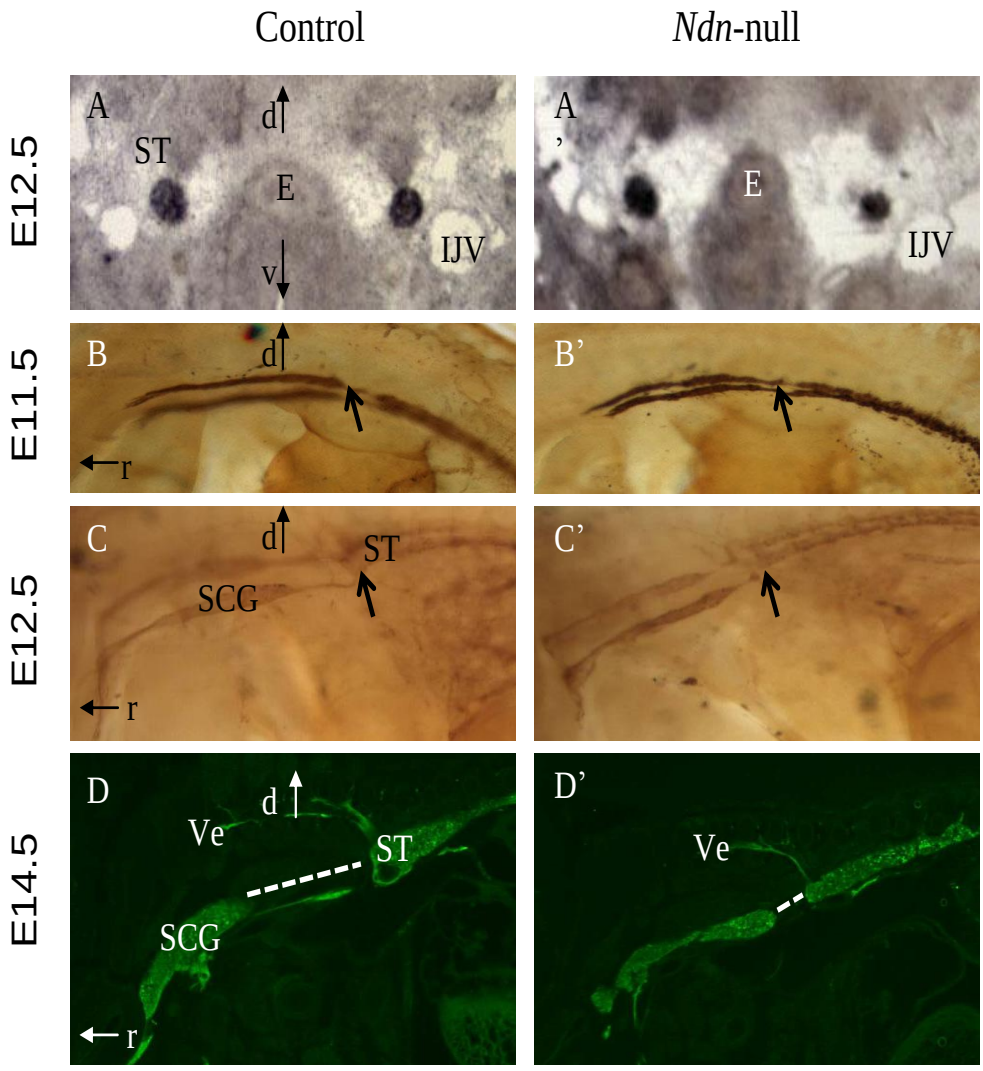

% Area relative to control mean
150
100
50
0
C2
C3
C4
C5
C6
C7
Ganglion location
Control
Ndn-null

Figure 2-1. Necdin is required for rostral neuronal migration and survival of superior cervical ganglia (SCG) neurons.

A) Thionine staining of 30μm parasagittal sections of E18.5 embryos oriented with the dorsal aspect (d) at the top and rostral (r) to the left. Arrowheads indicate the bifurcation of the carotid artery, arrows mark the SCG. C, cochlea. A') The SCG in *Ndn*-null embryos are located in a more caudal position than in the control littermates. B) Whole-mount anti-tyrosine hydroxylase (TH) immunohistochemistry (IHC) of E16.5 embryos, with the dorsal aspect toward the top and rostral to the left. Arrowheads indicate the stellate ganglion. The dashed lines approximately outline the SCG. B') The SCG in this typical *Ndn*-null embryo is identified by its expression of TH but extends abnormally caudally. C) Relationship of cell number of the SCG at E18.5 to its rostrocaudal location, expressed as the closest vertebral level to the middle of the ganglion. The two measurements indicated for each of four *Ndn*-null and control littermate embryos (eight measurements total) are from the right and left SCG. The SCG are 45% smaller in *Ndn*-null embryos than the control littermates ($P < 0.0001$), are located more caudally, and have greater variability in their final position between the left to right ganglia.

We then examined the earliest stages of development of sympathetic neurons, to determine the timing of SCG precursor migration abnormalities in *Ndn*-null mice. Phox2B is a transcription factor required for the differentiation and expression of TH in noradrenergic sympathetic neurons (Goridis and Rohrer 2002). Using RNA *in situ* hybridization with an antisense probe to Phox2B, we detected SCG precursors in the correct position in all E12.5 embryos, indicating that the initial specification and ventral migration of these cells is not compromised (Figure 2-2A). We then performed TH IHC on cryosections and whole embryos between E11.5 and E16.5, to follow the progression of the SCG neurons as they migrate rostrally and extend axons toward their target tissues. At E11.5, the most rostral column of cells in the sympathetic chain, the SCG precursors, appears similar in *Ndn*-null embryos compared with control littermate embryos (Figure 2-2B), although we did note a shortened column of SCG precursors in some *Ndn*-null embryos. At E12.5, there was reduced migration of a subset of SCG neurons but no difference in size of the SCG in *Ndn*-null embryos (Figure 2-2C). By E14.5, the SCG neurons in control littermate embryos have reached their final location between vertebral levels C1 and C4. In contrast, the *Ndn*-null SCG have an elongated shape and extend from C1 to C7, directly above the stellate ganglia (Figure 2-2D). In *Ndn*-null embryos, the SCG are also smaller at E14.5 compared with control littermates (83 ± 7% of control, P=0.02). In contrast to the continued rostral migration of SCG neurons in the wild-type embryos, no further migration of SCG neurons in *Ndn*-null embryos was observed from E14.5 to E18.5 (Figure 2-1A,B). This suggests that necdin is dispensable for dorsal-ventral migration of neural crest sympathetic precursors but is required for proper caudal-rostral migration of a subset of SCG neurons during embryonic development.


Control
Ndn-null
E12.5
E11.5
E12.5
E14.5
A
ST
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E
v
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E
IJV
B
d
r
B'
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d
ST
SCG
r
C'
D
d
Ve
ST
SCG
r
D'
Ve

Figure 2-2. Normal dorsoventral but abnormal rostral migration of sympathetic precursors during embryogenesis.

A) RNA in situ hybridization with a digoxigenin-labeled antisense probe to Phox2b was performed on transverse sections at E12.5 and detects neurons of the sympathetic trunk (ST). E, esophagus; Ijv, Internal jugular vein; d, dorsal; v, ventral. A') The sympathetic trunk expressing Phox2b is comparable in placement and size in the *Ndn*-null embryo, which indicates that the specification and ventral migration of neural crest sympathetic precursor cells proceeds normally. B) Whole-mount tyrosine hydroxylase (TH) immunohistochemistry (IHC) at E11.5. Dorsal is toward the top and rostral to the left in B-D. Note that the embryos in B and C are cleared to visualize both the left and right sympathetic trunk, with one side out of focus in the image. Arrows indicate the C7 vertebral level. B') Formation of sympathetic trunk is comparable between *Ndn*-null and control littermate embryos at E11.5. C) Whole-mount TH IHC (brown) at E12.5. Arrows indicate the nascent stellate ganglia (ST). C') The distance between the migrating superior cervical ganglia (SCG) and the ST is now smaller in the *Ndn*-null mouse, and a group of cells between the SCG and ST are already delayed in their rostral migration. D) Parasagittal cryosections (30μm) at E14.5 immunolabeled for TH (green). The SCG is located at the bifurcation of the carotid artery in the control embryo and has a characteristic ganglion shape. The white lines depict the interval between the SCG and the ST. The vertebral nerve (Ve) that projects from the stellate ganglion is shorter in the absence of necdin. H, heart. D') The SCG in *Ndn*-null embryos typically extend to the C7 vertebral level and have an elongated shape.

2.3.2 Ndn-null SCG Have Reduced Innervation of Target Tissues and Display Increased Apoptosis

The reduced size of the migrating *Ndn*-null SCG prompted us to examine if increased cell death is observed in these neurons. We identified apoptotic cells by immunostaining with an antibody to cleaved caspase-3, and identified the ganglia by co-labeling the cryosections with neuron-specific antibodies (anti-βIII-tubulin at E12.5 and anti-neurofilament at E14.5 and E18.5). At E18.5, the percentage of cells in the SCG of *Ndn*-null embryos labeled with cleaved caspase-3 was higher by 1.5-fold (control 1.1 ± 0.4%, *Ndn*-null 1.6 ± 0.5%; $P = 0.01$) (Figure 2-3). No significant difference was observed at E14.5 (control 0.16 ± 0.02%, *Ndn*-null 0.18 ± 0.03%), and cell death was not detected at E12.5 in either genotype. Cell proliferation was measured by immunolabeling with an antibody to Ki67, which is expressed in all proliferating or mitotic cells. No difference was observed in the percentage of proliferating cells at E12.5 (control 35 ± 4%, *Ndn*-null 32 ± 6%) or at E14.5 (control 21 ± 2%, *Ndn*-null 23 ± 3%). However, we did observe a small increase in proliferation at E16.5 in the *Ndn*-null embryos (control 19 ± 0.5%, *Ndn*-null 22 ± 2%; $P = 0.02$).

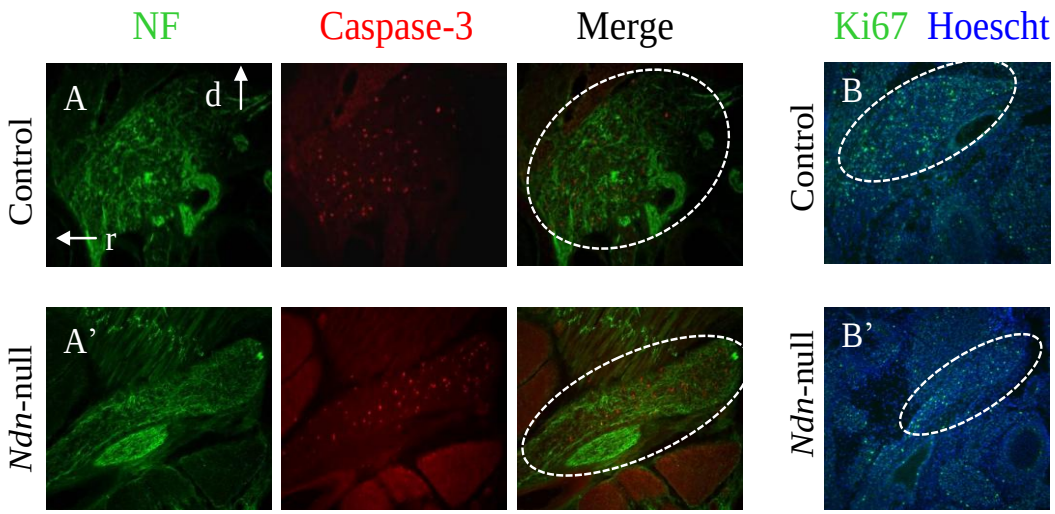
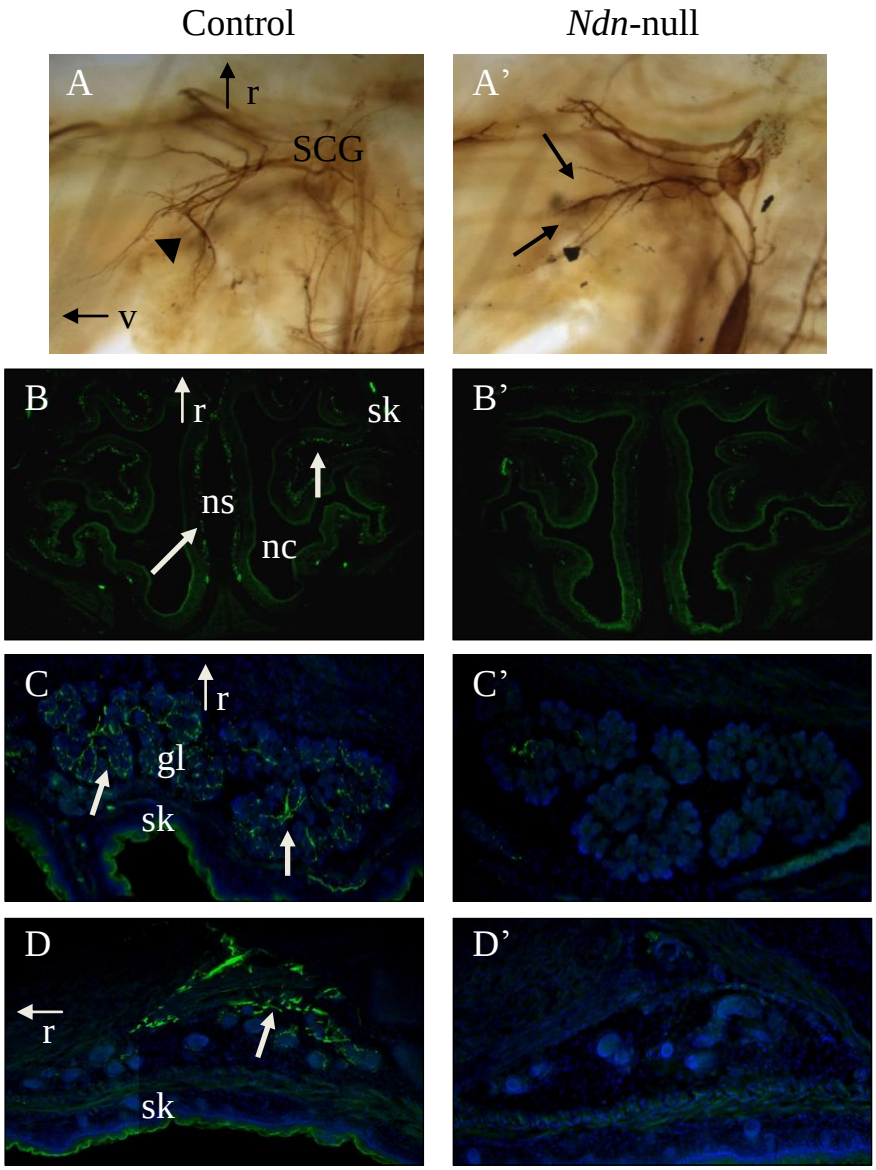


Figure 2-3. Cell death and proliferation in the *Ndn*-null SCG.
A,A') Representative 25µm parasagittal sections at E18.5 co-labeled with antibodies to neurofilament (NF) and cleaved caspase-3 (detecting apoptotic cells). B,B') Representative parasagittal sections at E14.5 labeled with an antibody to Ki67 (detecting proliferating cells, green) and stained with Hoescht stain (blue) to identify cell nuclei. The superior cervical ganglion (SCG) is outlined with a white dotted line and is more elongated in the *Ndn*-null embryo

We then examined axonal outgrowth and innervation of target tissues by the SCG by performing TH immunohistochemistry on whole mount embryos and cryosections at E18.5 (Figure 2-4). The SCG neurons normally innervate the parotid and subma.ndibular glands, nasal mucosa, and pupillary muscle. Outgrowth, branching and bundling of axons from the SCG is altered in *Ndn*-null embryos (Figure 2-4A), which results in a large reduction in innervation of both salivary glands and of the nasal mucosa (Figure 2-4B-D). We noted greatly reduced innervation of both salivary glands and of the nasal mucosa, with less innervation of the parotid gland than of the submandibular gland (2.8% and 18% of control littermate, respectively; Figure 2-4). The glands themselves were of comparable size in wild-type and *Ndn*-null embryos, and the submandibular ganglia were of normal position and size.



E	**Control**	**Necdin-null**
% Innervation Submandibular gland (mean ± SD)	2.2 ± 0.6	0.4 ± 0.07
% Innervation Parotid gland (mean ± SD)	3.3 ± 0.3	0.1 ± 0.03

Figure 2-4. Reduced innervation of superior cervical ganglia (SCG) targets is associated with defects in axonal outgrowth and branching.

A) Whole-mount tyrosine hydroxylase (TH) immunohistochemistry (IHC) of E16.5 embryos. Note that the embryo is cleared to visualize both the left and right sympathetic trunk, with one side out of focus in the images. Rostral is toward the top and ventral is toward the left. In the control embryo, the nerve from the SCG extends branches to innervate the sublingual glands as indicated by the arrowhead. A') In the *Ndn*-null embryo, there is a lack of branching and reduced extension of neurites as indicated by arrows. B-D) Representative 25-30μm transverse cryosections of E18.5 embryos were labeled with an antibody to TH. B,B') Nasal mucosa. ns, nasal septum, nc, nasal cavity, sk, skin. Rostral (r) is toward the top. C,C') Submandibular gland. gl, glandular tissue, sk, skin. Rostral (r) is toward the top. D,D') Parotid gland. sk, skin. Rostral (r) is to the left. E) Quantification of reduced innervation in *Ndn*-null salivary glands. Innervation of the parotid gland is reduced 35-fold and innervation of the submandibular gland is reduced 8-fold.

2.3.3 Examination of the Caudal Sympathetic Chain Ganglia, Parasympathetic and Enteric Nervous Systems

We identified a marked impairment in the migration and axonal outgrowth of SCG neurons in *Ndn*-null embryos, which prompted us to investigate the remaining sympathetic ganglia located in the thoracic and lumbar regions of the embryo. The sympathetic ganglia located caudal to the SCG are formed by the dorsal-ventral migration of trunk neural crest cells, which are a different group of neural crest cells from those that form the SCG. In contrast to SCG neurons, the neurons of the more caudal sympathetic chain ganglia do not migrate in a caudal-rostral direction. We performed whole-mount TH IHC on *Ndn*-null and control littermate embryos at E16.5 and E18.5 to examine the intact sympathetic nervous system in situ. We observed no difference in the size of the stellate ganglion, which is the first ganglion in the thoracic region of the sympathetic chain (Figure 2-5A). Of interest, the vertebral nerve from the stellate ganglion projects in the correct direction but is consistently shorter in *Ndn*-null embryos when examining both serial cryosections and whole-mount embryos (Figure 2-5B). The more caudal thoracic and lumbar sympathetic ganglia are normal in size and position (Figure 2-5A). The celiac ganglion and the mesenteric ganglia complex are correctly located in the lumbar region of the body, although axonal extensions are shorter in many *Ndn*-null embryos (Figure 2-5C). We noted reduced innervation of intestinal loops and punctate TH-positive staining in the gut (Figure 2-5D). This punctate TH staining is similar to what we observed to be associated with neurofilament-positive dystrophic spheroidal structures in the brainstem of *Ndn*-null mice (Ren et al. 2003; Pagliardini et al. 2005). TH-positive axons innervating the heart of *Ndn*-null embryos also contained punctate structures and were less arborized compared with wild-type (Figure 2-5E).

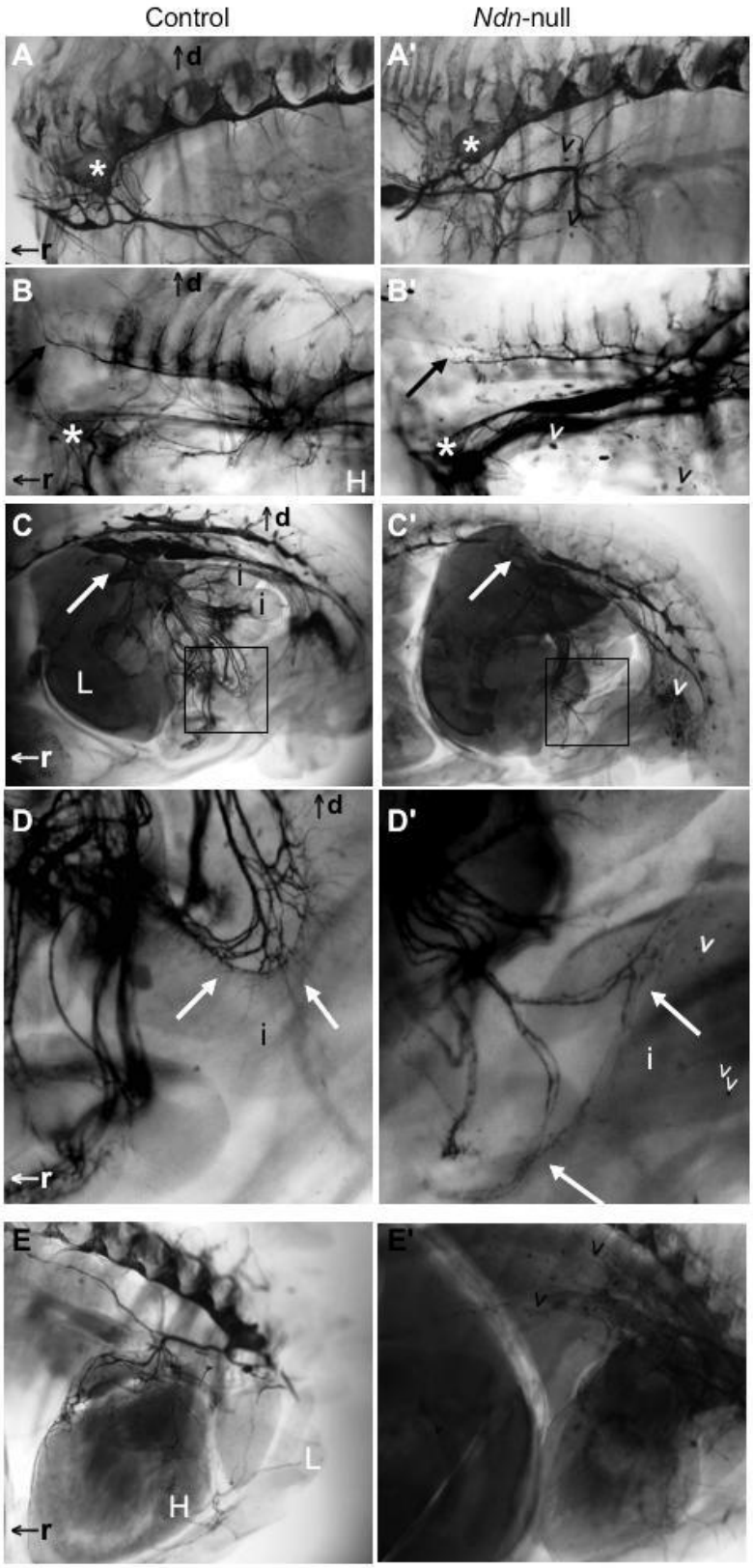

Control
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Figure 2-5. Loss of necdin does not affect the specification or localization of the remaining sympathetic chain ganglia but impairs neurite outgrowth.

Whole-mount tyrosine hydroxylase (TH) immunohistochemistry (IHC) of late-stage embryos. Note that the embryos are cleared to visualize both the left and right sympathetic trunk, with one side out of focus in the images. Dorsal (d) is toward the top and rostral (r) is toward the left. Arrowheads mark punctate TH-positive varicosities in the *Ndn*-null embryos. A,A') The stellate ganglion (asterisk) and more caudal sympathetic ganglia in the thoracic region are comparable in size and location in *Ndn*-null and control embryos at E18.5. The heads have been removed from these embryos. B) Whole-mount TH IHC of E16.5 embryos. Arrow indicates the terminus of the vertebral nerve projecting from the stellate ganglia (STG). B') The vertebral nerve that projects from the stellate ganglion is shorter in the absence of necdin. C,C') Whole-mount anti-TH IHC of E18.5 embryos. The prevertebral sympathetic ganglion complex, which includes the inferior and superior mesenteric ganglia, and the celiac ganglion are similarly formed in *Ndn*-null and control littermates. However, sympathetic innervation of the intestinal tract (i) is visibly reduced in the *Ndn*-null embryo, with shorter and less branched axons (inset, see D,D'). L, liver. Arrow indicates the celiac ganglion complex. D,D') Inset from C demonstrating reduced innervation of intestinal loops (arrows). E,E') Innervation of the heart (H) by the thoracic ganglia is also compromised in *Ndn*-null embryos and punctate varicosities are seen. L, liver.

Ret tyrosine kinase is a transmembrane receptor expressed by neurons originating from vagal neural crest cells, which includes the SCG and enteric neuron precursors; and by developing renal cells. Therefore, we used *Ret* tyrosine kinase RNA in situ hybridization on parasagittal sections of E12.5 embryos to examine the development of the enteric nervous system in *Ndn*-null embryos. We detected normal expression in the sympathetic chain ganglia, kidneys, and enteric nervous system in *Ndn*-null embryos, suggesting that neural crest progenitors can adequately populate the gastrointestinal tract (Figure 2-6). As we now have *Ndn*-null mice that survive into adulthood, I performed acetylcholinesterase staining of various portions of the small intestines to support the observation of a normal developing ENS in the *Ndn*-null embryos. Preliminary investigation of the gross innervation pattern was similar between genotypes, supporting the notion that necdin is not required for the migration of enteric neurons (Figure 2-7).

Three cranial parasympathetic ganglia (sphenopalatine ciliary, and otic) were also examined using an antibody to choline acetyltransferase (ChAT) but no gross defects were identified in the number of neurons present or the expected location of the ganglia (Figure 2-8). The adrenal glands were the same size in both genotypes (*Ndn*-null 96 ± 12% of control) and had similar numbers of cells expressing TH (control 7 ± 2%, *Ndn*-null 6 ± 1%). In summary, many sympathetic neurons examined had some degree of defective axonal extension. Nonetheless, the neuronal precursors derived from the vagal region of the spinal cord and that undergo rostral migration are preferentially affected by the loss of necdin, compared with those derived from the trunk neural crest.

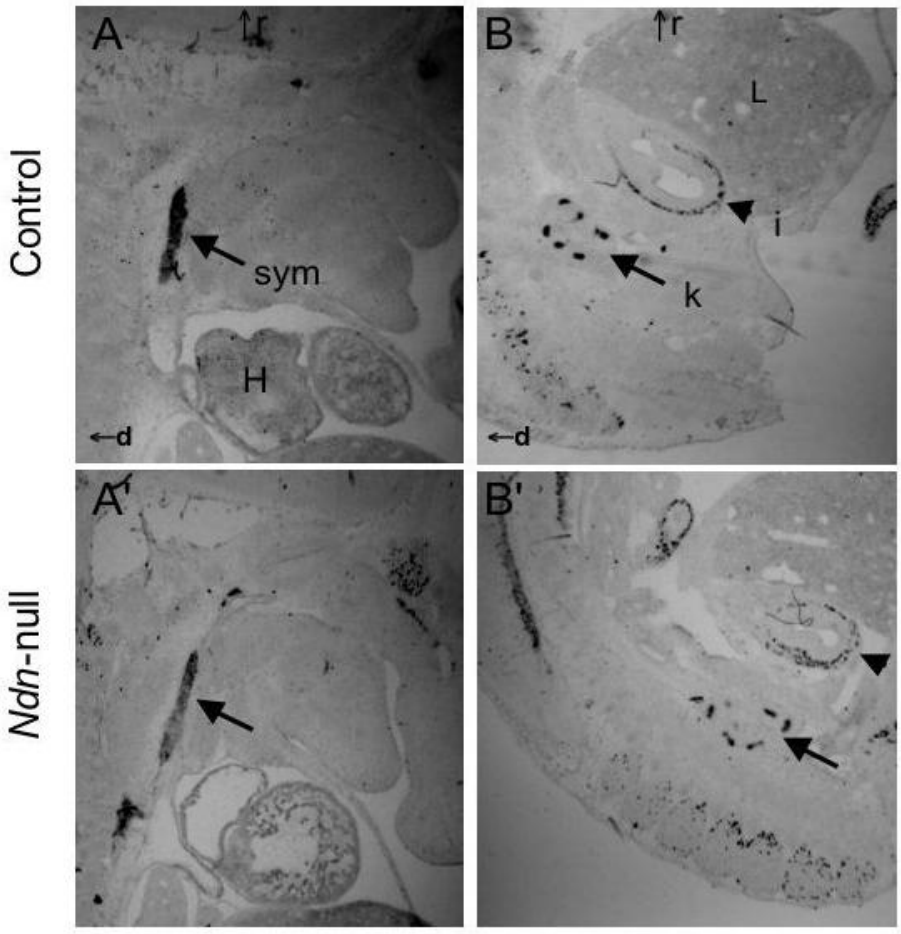

Figure 2-6. Normal expression of Ret tyrosine kinase mRNA in the sympathetic chain ganglia, enteric nervous system and kidneys of *Ndn*-null embryos.
RNA *in situ* hybridization with a digoxygenin (DIG)-labeled antisense probe to Ret on parasagittal sections at E12.5 in control (A, B) and *Ndn*-null (A',B') embryos. A,A') Ret expression in the sympathetic chain is marked with an arrow. H, heart. B,B': Ret expression in the intestine (arrowhead) and kidney (arrow). Dorsal is toward the left, rostral toward the top. L, liver.

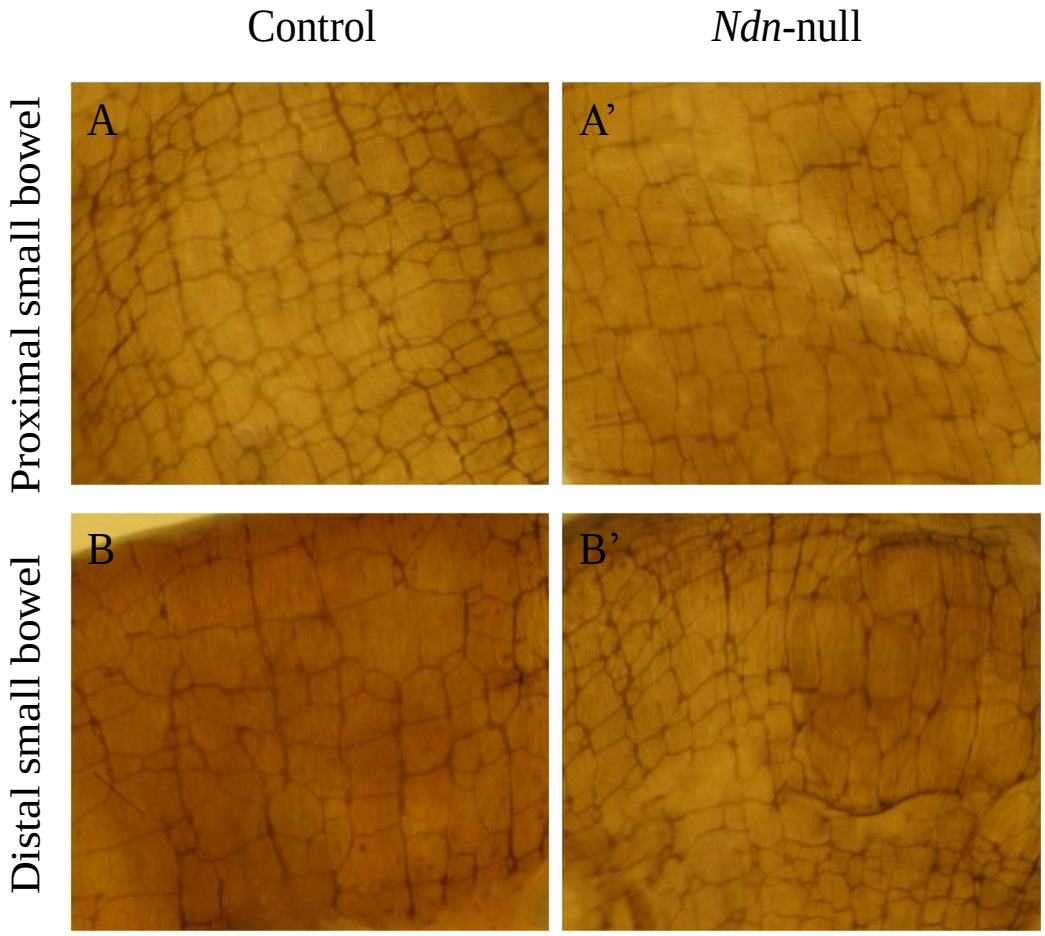

Figure 2-7. Loss of necdin does not affect the development of enteric neuron networks in small intestines.

Acetylcholinesterase staining of enteric neurons in the myenteric and submucosal plexuses in whole-mount small intestines in control and *Ndn*-null mice. (A,A') Representative images of the proximal small bowel in control and *Ndn*-null mice. (B,B') Representative images of the distal small bowel in control and *Ndn*-null mice.

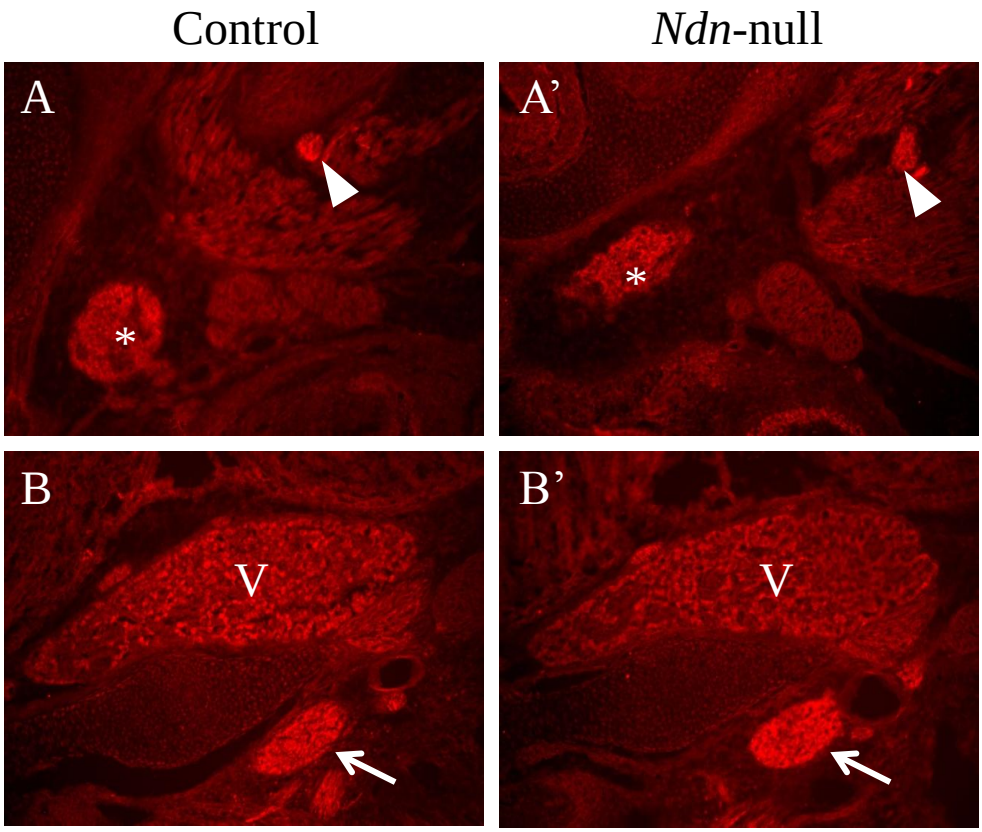

Figure 2-.8. Necdin is not required for the localization of cranial parasympathetic ganglia.

Representative 25μm coronal sections at E18.5 labeled with an antibody to choline acetyltransferase (ChAT). (A, A') The arrowhead indicates the ciliary ganglion and the asterisk indicates the sphenopalatine ganglion. Note that these ganglia are comparable in size and location in the *Ndn*-null embryo. (B, B') The arrow indicates the otic ganglion which is located directly caudal to the trigeminal (V) ganglion. Both are comparable in size and location in the *Ndn*-null embryo.

2.4 Discussion

Loss of necdin had previously been shown to cause defects in the survival and neurite outgrowth of central and sensory neurons. We have shown that loss of necdin in mice also impairs the survival and axonal elongation of sympathetic neurons and greatly reduces innervation of target glands, preferentially affecting the most rostral sympathetic ganglia. Unexpectedly, loss of necdin also greatly impairs the migration of SCG neurons in late embryogenesis, presenting a previously unknown cellular role for necdin. Significant deficits in the formation of other sympathetic chain ganglia were not observed, suggesting that necdin is particularly important for the rostral migration and survival, but not proliferation, of SCG neurons. The neurons that migrate to form the enteric nervous system also migrate a considerable distance into the gastrointestinal tract, but are normally located in the absence of necdin. The ventral migration of sympathetic precursors from the region near the dorsal neural tube to the aorta is also unaffected by the loss of necdin.

The migration of neurons is a universal feature in the development of the nervous system and ultimately requires rearrangement of the cytoskeleton, which is regulated by intracellular signaling pathways that induce cytoskeletal reorganization coordinated by the centrosome/microtubule organizing centre (Badano et al. 2005; de Anda et al. 2005). One important feature of cellular migration is the movement of the centrosome into the region between the nucleus and the leading edge, which determines the direction of migration, and requires an extensive amount of cytoskeletal rearrangement. Notably, axonal outgrowth also requires cytoskeletal rearrangement coordinated with the direction of migration (de Anda et al. 2005). Many proteins have been identified that regulate the cytoskeleton and associated molecular motors during these dynamic processes (Badano et al. 2005). We previously identified interactions between necdin and two proteins important in neuronal cytoskeletal rearrangement, namely fasciculation and elongation protein zeta-1 (Fez-1) and Bardet-Biedl syndrome 4 (BBS4) (Lee et al. 2005). Bardet-Biedl syndrome, like PWS, is genetic syndrome

with obesity and developmental delay, but differs from PWS in that it is associated with ciliary dysfunction. Specifically, BBS4 recruits proteins to the pericentriolar region and is required for the anchoring of microtubules and cell cycle progression (Kim et al. 2004). Fez1 is essential for the activation of the microtubule-based molecular motor kinesin-1, but was first described for its role in axon bundling and outgrowth (Bloom and Horvitz 1997; Blasius et al. 2007). We previously observed varicosities in irregularly oriented axons of both serotonergic and motor neurons in *Ndn*-null embryos, and deficient neurite outgrowth was observed in explants of dorsal root ganglia at E13.5 (Kuwako et al. 2005). We now report similar neurite abnormalities in sympathetic neurons, manifesting as tyrosine hydroxylase positive varicosities present where the heart and intestinal tract are innervated by sympathetic chain neurons. Thus, accumulating evidence suggests that loss of necdin impairs the cytoskeletal program required for the coordination of neurotrophin signaling and axonal outgrowth, and now additionally for cellular migration.

The survival of specific sets of embryonic neurons is also compromised by *Ndn*-deficiency. In *Ndn*-null dorsal root ganglia, increased cell death occurs between E12 and E14 (Kuwako et al. 2005; Andrieu et al. 2006), while activity-deprivation related apoptosis is increased in postnatal *Ndn*-null cerebellar granule cells (Kurita et al. 2006). In this study, we observe increased apoptosis in *Ndn*-null sympathetic neurons at E18.5. Notably, decreased survival coincides with developmental time points during which target-derived neurotrophic support and intact neurotrophin signaling are essential for survival. The neurotrophic requirements of the superior cervical ganglia (SCG) neurons have been most finely delineated through gene-targeting in mice. For example, deletion of the gene encoding the neurotrophin NT-3 causes deficits in proximal axon extension that lead to reduced innervation of sympathetic targets and a 50% loss of SCG (Ernfors et al. 1994; Kuruvilla et al. 2004). Ablation of glial cell line-derived neurotrophic factor signaling through loss of the Ret tyrosine kinase gene causes defective migration and axonal outgrowth of the SCG, other sympathetic chain ganglia, and the ENS (Enomoto et al. 2001). In view of the dual role of

neurotrophic factors in promoting neurite outgrowth and supporting neuronal survival, we favour a hypothesis that the limited availability of target-derived neurotrophic growth factors and reduced signaling through neurotrophin receptors combine to impede neurite outgrowth, and reduce the survival of late embryonic *Ndn*-null neurons. In summary, we propose that necdin plays a critical role in the interrelated processes that link growth factor-responsive intracellular signaling pathways with the cytoskeletal rearrangements required for cellular migration during development.

Necdin is a member of the type II MAGE (melanoma antigen) family of proteins, which have distinct roles in apoptosis and cell cycle progression in nervous system development (Barker and Salehi 2002). Notably, three MAGE proteins (NRAGE/MAGED1, MAGEH1, and MAGEG1) also interact with $p75^{NTR}$ and may have overlapping functions in the development of the nervous system, providing some functional redundancy in specific neuronal subsets (Salehi et al. 2000; Salehi et al. 2002; Tcherpakov et al. 2002; Kuwako et al. 2005; Kurita et al. 2006). NRAGE is expressed in the superior cervical ganglia and the sympathetic chain (Kendall et al. 2002). We then reviewed the expression profiles presented in an analysis of expressed sequence tag counts, using the National Center for Biotechnology Information EST Profile Viewer. Only necdin, MAGED2, and MAGEE1 are represented among 10,966 transcripts derived from murine sympathetic ganglia cDNA libraries. These three genes and MAGED1 are also more widely expressed in the brain and other parts of the nervous system.

People with Prader-Willi syndrome have a congenital absence of necdin (Jay et al. 1997; MacDonald and Wevrick 1997; Sutcliffe et al. 1997). We previously proposed that defects in axonal extension and arborization in the developing central nervous system could be implicated in cognitive and respiratory compromise in people with PWS (Ren et al. 2003; Pagliardini et al. 2005). We observed reduced innervation of the salivary glands in *Ndn*-null embryos, which if present in people with PWS, could account for the observed

abnormalities in saliva output that generate adverse effects, most notably difficulty with swallowing (Hart 1998). A defect in parasympathetic innervation of the gut has previously been described as a possible reason for gastrointestinal impairments observed in PWS (Goldstone 2004). We did not observe such innervation defects in the gut of *Ndn*-null embryos, nor did we find defects in the population of the gut by enteric neurons, but sympathetic innervation of the intestinal tract was compromised. Given the complexity of the MAGE protein family, it is likely that functional redundancies in mice may not completely mirror those that are present in humans. Notably, the MAGE protein Magel2 is also congenitally absent in people with PWS (Boccaccio et al. 1999; Lee et al. 2000). *MAGEL2* is highly expressed in the hypothalamus, which conveys information about autonomic status to the peripheral autonomic system through the lateral medulla. It is possible that the combined loss of necdin and Magel2 could have a more severe impact on autonomic function than loss of necdin alone, through combined central and peripheral autonomic dysfunction. Alternatively, the loss of necdin function in the smooth muscle of the intestine could contribute to gastric dysmotility independent of autonomic dysfunction. Further studies that examine whether there is reduced innervation or decreased smooth muscle function in the gastrointestinal tract in people with PWS, and that examine the effect of the concurrent loss of necdin and Magel2 in mice are needed to further test these hypotheses.

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Chapter 3. *Magel2*-null mice have altered stress responses, growth hormone deficiency, and a delayed counter-regulatory response to hypoglycemia suggestive of hypothalamic deficiency.

Parts of this chapter have been submitted for publication June 24, 2010.

(Endocrinology: EN-10-0709 Version 1)

With the exception of the high fat diet glucose tolerance test, Alysa Tennese completed all the experiments presented in this chapter. This testing was performed at the University of Cincinnati Mouse Metabolic Phenotyping Center.

3.1 Introduction

Prader-Willi syndrome (PWS) is a complex genetic disorder, with clinical features that suggest functional abnormalities of the hypothalamic-pituitary axis. These include short stature, growth hormone (GH) deficiency, hypogonadotropic hypogonadism, childhood-onset hyperphagia and obesity, and sleep disorders. Structural abnormalities of the brain have been documented in PWS, including frequent ventriculomegaly and cortical abnormalities. Pituitary abnormalities are relatively common, but structural abnormalities of the hypothalamus are not typically found (Miller et al. 1996; Schmidt et al. 2000; Miller et al. 2007; Iughetti et al. 2008). Short stature compared to family members is a cardinal feature in PWS individuals not treated with GH. Children with PWS typically have decreased spontaneous GH secretion, and low peak GH response to stimulation tests (Theodoridis et al. 1971; Burman et al. 2001), accompanied by reduced serum insulin-like growth factor 1 (IGF-1) and low IGF-binding protein 3 (Eiholzer et al. 1998; Miller et al. 2008; Sode-Carlsen et al. 2010). Relative GH deficiency contributes to abnormal body composition consisting of increased body fat mass and reduced lean mass, and treatment with GH partially normalizes body composition (Eiholzer et al. 2004; Mogul et al. 2008; Carrel et al. 2010). In contrast, obesity in the general population is often associated with a relative GH deficiency but normal or high levels of IGF-1, suggesting defective feedback mechanisms that can resolve on weight loss.

In normal adults, serum leptin levels increase with increasing adiposity, but levels of adiponectin, particularly in its multimeric high molecular weight form, are inversely correlated with fat mass (Hu et al. 1996; Yamauchi and Kadowaki 2008). Lower adiponectin levels are typically found in type 2 diabetics and correlate with insulin resistance in obese controls (Saltiel 2001). Adiponectin acts primarily through receptors present in liver and in muscle (Yamauchi and Kadowaki 2008), but also acts in the paraventricular nucleus of the hypothalamus to stimulate food intake and decrease energy expenditure during fasting (Qi et al. 2004; Kubota et al. 2007). Obese individuals with PWS have serum leptin levels

expected from their degree of adiposity. However, despite their increased adiposity, PWS plasma adiponectin levels are higher than those in obese controls, but lower than or similar to those of lean individuals (Hoybye et al. 2004; Pagano et al. 2005; Kennedy et al. 2006; Festen et al. 2007; Haqq et al. 2007). Consistent with higher adiponectin levels, obese adults with PWS are relatively insulin sensitive compared to obese controls (Bray et al. 1983; Schuster et al. 1996; Talebizadeh and Butler 2005).

Central adrenal insufficiency has recently been appreciated as contributing to sudden deaths in children with PWS, including cases of mild or moderate upper respiratory tract infections (Rudd et al. 1969; Schrander-Stumpel et al. 2004; Stevenson et al. 2004; de Lind van Wijngaarden et al. 2008). One recent study used an overnight single-dose metyrapone test to inhibit cortisol production, stimulating adrenocorticotropic hormone (ACTH) production. About 60% of tested PWS individuals showed an insufficient ACTH response in the metyrapone test, demonstrating central adrenal insufficiency (de Lind van Wijngaarden et al. 2008). The frequency of hypothyroidism in the PWS population is reported to be up to 24%, with various studies reporting normal thyroid hormone and baseline thyroid stimulating hormone (TSH) (Butler et al. 2007), low T_4 with high T_3 levels (Festen et al. 2007), low free T_4 and low/normal TSH in 19% of PWS individuals (Miller et al. 2008), or hypothyrodism in 24% of French children with PWS (Diene et al. 2010).

The *MAGEL2* gene is one of several imprinted genes that are typically inactivated in people with Prader-Willi syndrome (Boccaccio et al. 1999; Lee et al. 2000). In the mouse, *Magel2* is most highly expressed in the hypothalamus, with maximal levels in the suprachiasmatic nucleus, which controls circadian rhythm, and in the arcuate nucleus, which controls energy homeostasis (Lee et al. 2000). The encoded protein MAGEL2/Magel2 is a member of the MAGE family of proteins, which act as adaptor proteins for complexes that participate in the transduction of extracellular signals (Barker and Salehi 2002). We previously described decreased activity, progressive infertility, and blunted circadian rhythm

in mice lacking *Magel2* (Kozlov et al. 2007; Mercer and Wevrick 2009). We also found that *Magel2*-null mice are obese, with increased body fat, increased serum leptin, and decreased lean mass, but normal length (Bischof et al. 2007). We now report endocrine dysfunction in *Magel2*-null mice, including profound insulin-induced hypoglycemia, a marked delay in the counter-regulatory hypoglycemia response, relative insulin sensitivity, and increased adiponectin. We also discovered abnormalities of the hypothalamic pituitary axes in *Magel2*-null mice, including elevated basal corticosterone levels, reduced GH release, and reduced Igf-1 levels. Our work suggests that Magel2 is essential for the normal feedback mechanisms that regulate endocrine axes controlled by the hypothalamus.

3.2 Materials and Methods

Mouse breeding, genotyping, and housing

All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Animal Policy and Welfare Committees for the University of Alberta. The *Magel2*-null mice have been maintained on a C57Bl/6 background for at least 15 generations and were genotyped as described (Bischof et al. 2007). Mice carrying a paternally-inherited lacZ-knockin allele are functionally null for *Magel2* and are referred to as *Magel2*-null; littermates that are wild-type for *Magel2* were used as controls. Mice were weaned between 3 and 4 weeks of age then housed 2–3 per cage with food and water *ad lib.*, and maintained under 12:12 light dark conditions. One set of mice was fed a high fat diet (Basal purified diet w/60% energy from fat, LabDiet) for 8 weeks after weaning. For tail vein blood collection, the time between blood samples was at least six days, with up to 100 µl (average 50 µl) blood collected each time.

Stress-induction protocol

Morning basal samples were collected between 9 and 11 a.m. All stress-induced experiments were performed between 8 a.m. and 12 p.m., which is the nadir of corticosterone release in rodents. Tail blood was collected before or after

a stressor from partially restrained mice. Restraint stress-induced samples were collected from mice restrained in 50 ml conical tubes for 30 minutes. Before testing for insulin-induced hypoglycemia, mice were fasted for two hours and human insulin (0.75 U/kg, Sigma-Aldrich, Oakville, ON, in 100 µl normal saline) was injected intraperitoneally. Blood glucose was measured by the glucose oxidase method (Contour Glucometer, Bayer, Canada) after 0, 15, 30, 60 and 120 minutes, in a small drop of blood from the tail vein. A blood sample collected 30 minutes post-injection was tested for corticosterone. For the dexamethasone suppression test, mice were injected intraperitoneally with 0.15 mg/kg dexamethasone in 100 µl saline. Tail blood was collected 1 and 4 hours post-injection. For the ACTH stimulation test, mice were injected intraperitoneally with 25 mg/kg metyrapone, then corticosterone levels were measured after one hour and ACTH levels after 6 hours. For GH assays, mice were injected intraperitoneally with saline (control), 60 µg/kg growth hormone-releasing hormone (GHRH) (Sigma-Aldrich), or 120 µg/kg rat ghrelin (Tocris Bioscience, Ellisville, MO) in a final volume of 100 µl saline. Injections occurred between 1 and 5 p.m., and tail blood was collected 5-15 minutes post-injection.

Glucose Tolerance Testing

Following a six hour fast, mice fed a normal chow or high fat diet for 6 weekswere injected intraperitoneally with 1 mg/g glucose in 0.9% saline. Glucose levels were measured from a tail vein blood drop at 0, 5, 15, 30, 60, and 120 minutes. Blood (30 µl) was collected at 0 and 30 minutes for insulin determinations in the high-fat diet fed animals. Values for the homeostasis model assessment of insulin resistance (HOMA-IR) were calculated as fasting glucose (mmol/l) × fasting insulin (microunits/ml) divided by 22.5. A higher HOMA value indicates a higher level of insulin resistance. The high-fat diet testing was performed at the University of Cincinnati Mouse Metabolic Phenotyping Center.

Hormone assays

Collected blood was allowed to clot at room temperature for 30 min, and centrifuged at 2000 X g for 10 min in an Eppendorf 5417C microcentrifuge (Eppendorf Canada, Missassauga, ON). Serum was aliquoted and stored at -20°C until assayed for hormone content. Serum samples were analyzed using the following enzyme-linked immunosorbent assays (ELISAs): high sensitivity corticosterone (IDS Inc., Fountain Hills, AZ), ACTH (Calbiotech, Spring Valley, CA), Igf-1 (Antigenix America, Huntington Station, NY), GH (Millipore, Billerica, MA), T_4 (Calbiotech), and high molecular weight/total adiponectin (Alpco Diagnostics, Salem NH) according to manufacturers' instructions. Intra-assay and inter-assay variation data were provided by the manufacturers. Hormone levels were determined using the standardized reagents supplied in the kits to generate standard curves, with serum dilutions used to keep all measurements described within the range of the standard curve.

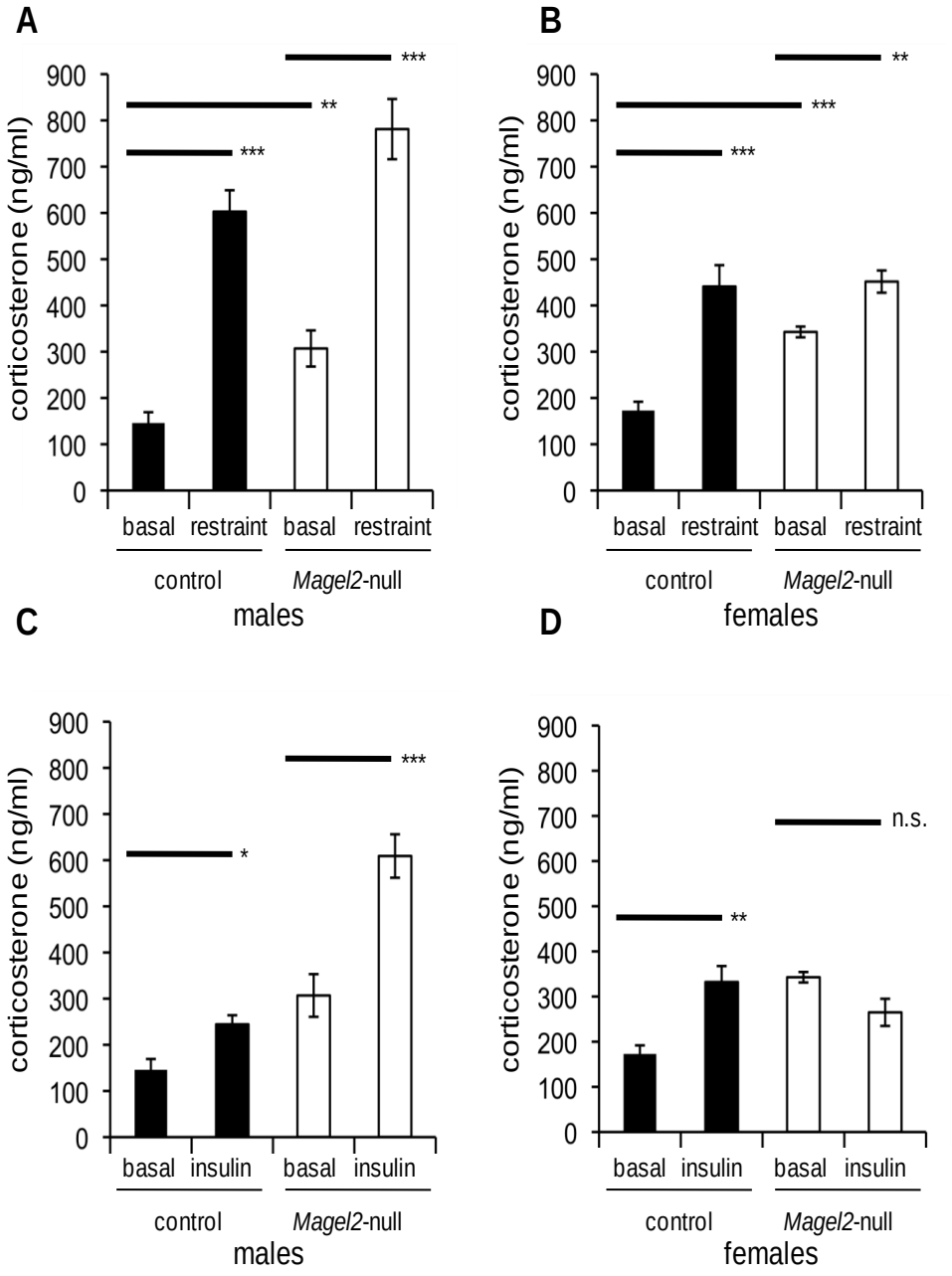
Statistical analysis

Results are expressed as the mean±SEM unless otherwise stated. Repeated measures analysis of variance was calculated within GraphPad Prism 4 software. Analysis of selected differences between genotypes or treatments was performed using a Student's t-test. Standard deviation scores (SDS) refer to the difference between the measurement and the control mean, divided by the control standard deviation (SD). A Mann-Whitney (non-parametric) test was used in instances where data was not normally distributed. A P-value <0.05 was considered statistically significant.

3.3 Results

3.3.1 Increased basal corticosterone levels and altered stress response in Magel2-null mice

We first noted that basal corticosterone levels were 2.1 and 2.0-fold higher in male and female *Magel2*-null mice respectively compared to control littermates ($P<0.01$) (Figure 3-1A,B). It was formally possible that the handling required for blood sampling was sufficient to induce a stress response in *Magel2*-null but not control mice, thus giving the appearance of high baseline corticosterone levels. To test this, we collected trunk blood samples from *Magel2*-null mice after sudden decapitation with minimal handling (n=4 of each genotype). Corticosterone levels in trunk blood were comparable to those observed in tail blood samples, suggesting that basal corticosterone levels are indeed elevated in the mutant mice. Stressful situations activate the hypothalamic-pituitary-adrenocortical (HPA) axis, causing a rapid increase in circulating glucocorticoid levels. Corticosterone levels were measured after 30 minutes of physical restraint. As expected, control mice demonstrated a significant increase over their basal levels following restraint stress (4.9±1.1-fold and 2.8±0.6-fold over basal in control male and female mice respectively, both significantly increased over basal, $P<0.0001$) (Figure 3-1A,B). Corticosterone levels also increased over baseline in response to restraint in *Magel2*-null mice (2.7±0.3-fold (*Magel2*-null male) and 1.3±0.1 fold (*Magel2*-null female) over basal, $P<0.002$). However, the magnitude of this response was decreased in the *Magel2*-null female mice compared to control ($P<0.05$).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females

Figure 3-1. *Magel2*-null mice have altered HPA axis responses to stress.

Values represent the mean±SEM, n=5-8 per genotype per sex. Black bars represent control mice, white bars represent *Magel2*-null mice. Serum corticosterone levels were determined at baseline, 30 minutes post-restraint and 30 minutes post-insulin injection. A) Basal levels of serum corticosterone are 2.1-fold elevated in *Magel2*-null male mice compared to control littermates. Restraint corticosterone levels are increased 4.9- and 2.8-fold in control and *Magel2*-null male mice respectively. B) Basal levels of serum corticosterone are 2.0-fold elevated in *Magel2*-null female mice compared to control littermates. Restraint corticosterone levels are increased 2.7- and 1.3-fold in control and *Magel2*-null female mice respectively. C) Serum corticosterone levels are elevated 2.0- and 2.1-fold in control and *Magel2*-null male mice respectively post-insulin injection. D) Serum corticosterone levels are elevated 2.0- and 0.9-fold in control and *Magel2*-null female mice respectively post-insulin injection. (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; n.s., not significant).

3.3.2 Delayed counter-regulatory response to hypoglycemia in Magel2-null mice

As a more specific test of the reactive stress response, we measured corticosterone levels 30 minutes after injection of a dose of insulin sufficient to cause hypoglycemia (0.75 U/kg). As expected, corticosterone levels in control mice increased an average of 2.0±0.3-fold (female) and 2.0±0.6-fold (male) over basal levels after 30 minutes, diagnostic of an intact HPA response (P<0.03, Figure 3-1C,D) (Erturk et al. 1998). Remarkably, *Magel2*-null female mice had no change in corticosterone levels after insulin administration, although an appropriate response (2.1±0.2-fold increase, P<0.001) was seen in the male *Magel2*-null mice.

We confirmed that the mice became hypoglycemic by sampling blood glucose levels at intervals up to 120 minutes after injection of insulin (Figure 3-2). Baseline blood glucose levels did not differ between genotypes in either sex. Control mice became hypoglycemic with a maximum mean decrease after 30 minutes to a minimum of 3.8±0.3 mmol/l (female mice) and 3.6±0.5 mmol/l (male mice) (Figure 3-2). This extent of hypoglycemia is sufficient to induce a robust corticosterone response in mice. We noted no significant difference between genotypes in the blood glucose lowering effect of injected insulin in the first 15 minutes. However, by 30 minutes, *Magel2*-null mice became more profoundly hypoglycemic than control mice, dropping to a minimum mean value of 2.1±0.3 mmol/l in males (range 1.3 to 2.9 mmol/l, P<0.02 compared to control) and 2.2±0.3 mmol/l in females (range 1.1 to 2.9 mmol/l, P<0.01 compared to control). The start of the return to euglycemia was delayed to 60 minutes post-injection, from a normal time of 30 minutes, in both male and female *Magel2*-null mice. We calculated the glucose recovery rate as a measure of the counter-regulatory response to hypoglycemia. In control female mice, blood glucose levels began to return to pre-injection glucose levels at a glucose recovery rate of 65±6 mmol/l/min. during the second hour. *Magel2*-null mice, particularly female mice, remained hypoglycemic up to 120 minutes post-injection, with a glucose recovery rate was reduced to 32±1 mmol/l/min (P<0.01 compared to control). Overall,

Magel2-null mice had a significantly enhanced response to injected insulin, both over the entire time course ($P<0.002$ by repeated measures ANOVA) and specifically at the one hour time point ($P<0.001$ by two-tailed t-test). However, the mice do recover from hypoglycemia without intervention, suggesting a delayed rather than deficient counter-regulatory response. To summarize, *Magel2*-null mice experienced a more profound hypoglycemia and delayed recovery from insulin-induced hypoglycemia compared to control littermates. Female mice are more severely affected, with a delay to 60 minutes in the return to euglycemia, and no change in corticosterone levels 30 minutes after insulin-induced hypoglycemia.

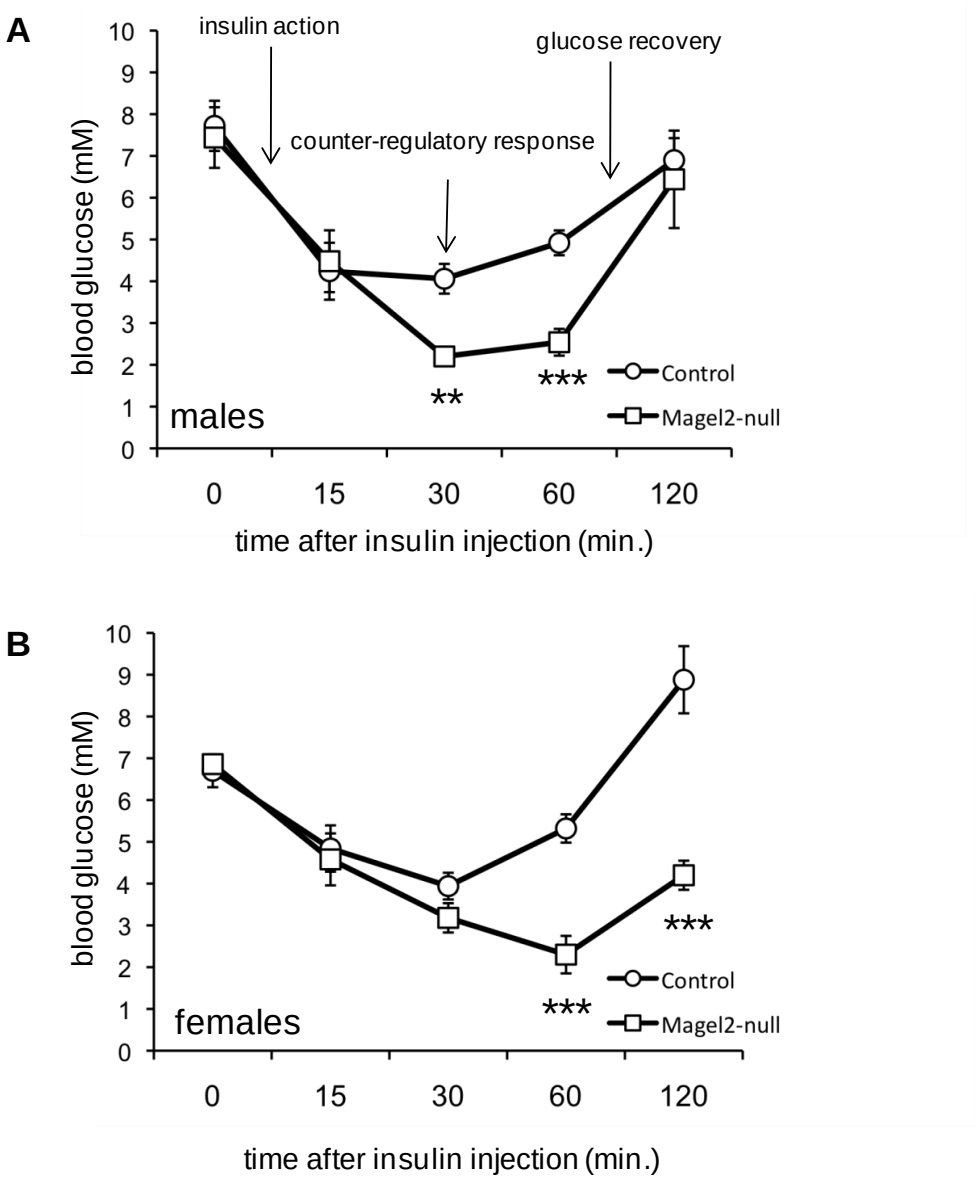
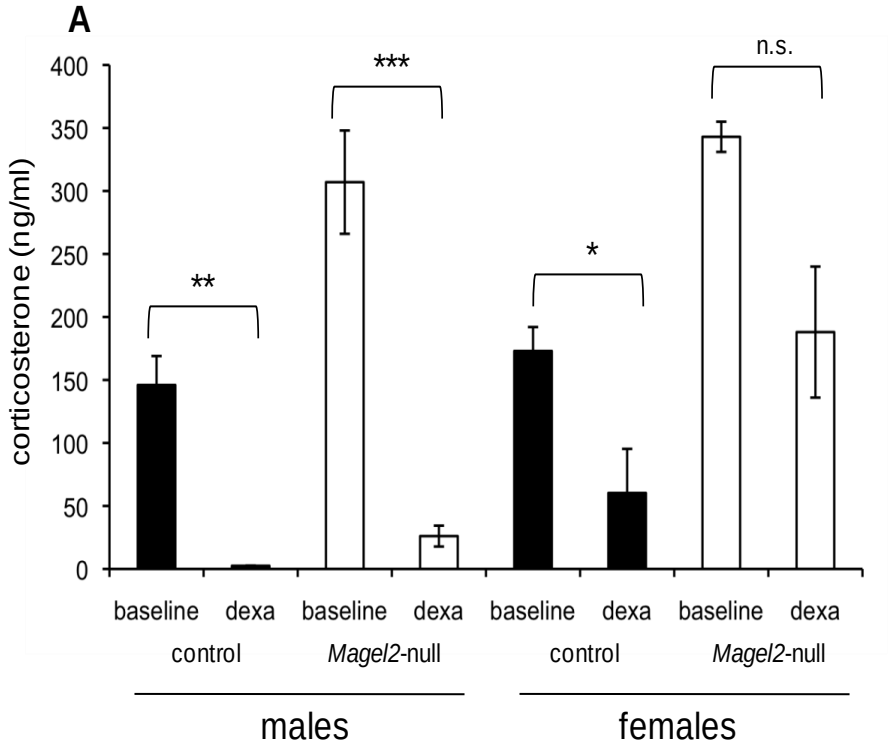
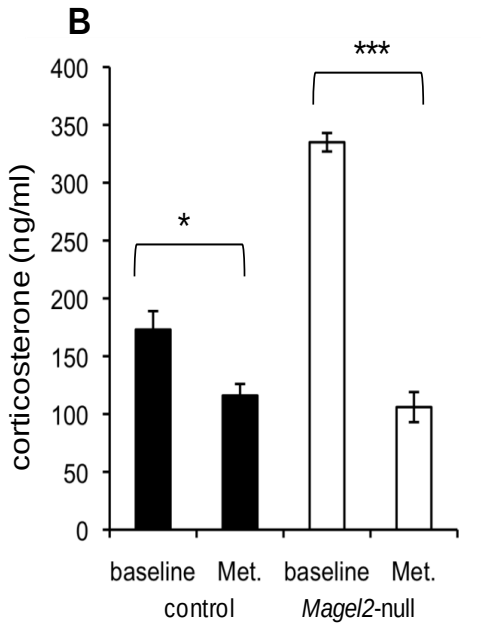


Figure 3-2. Insulin tolerance test (ITT) in control and *Magel2*-null mice. Values represent the mean±SEM, n=5 per genotype per sex. Mice were injected with 0.75U of insulin. A) Blood glucose levels were measured in control and *Magel2*-null male mice. B) Blood glucose levels were measured in female control and *Magel2*-null female mice. The minimum blood glucose level was reduced in both sexes, while the time to initiate recovery and the glucose recovery rate was reduced in *Magel2*-null female mice. (**, P<0.01; ***, P<0.001).

3.3.3 Hypothalamic-pituitary-adrenal axis responses to low or high corticosterone

The reduced responses to restraint and hypoglycemia suggest a deficit in the stress-induced glucocorticoid response. We next tested *in vivo* responses to acute changes in circulating corticosteroids, administering dexamethasone to test negative feedback regulation of the HPA axis, or metyrapone to test feedback regulation in response to a drop in corticosterone. Corticosterone levels measured after control saline injection were similar to basal levels, and were two- to three-fold elevated in the *Magel2*-null mice compared to control mice. Both sexes of control mice, and *Magel2*-null male mice, responded to a dexamethasone injection with a mean decrease in corticosterone measured after 1 and 4 hours (Figure 3-3A, 4 hour time point shown). However, mean corticosterone levels were not significantly lower in *Magel2*-null female mice after dexamethasone injection (Figure 3-3A). To further test the responsiveness of the HPA axis, we administered the 11β-hydroxylase inhibitor metyrapone, which inhibits glucocorticoid production, stimulating ACTH production (Giordano et al. 2008). Metyrapone effectively suppressed corticosterone levels measured one hour post injection, in both the control and *Magel2*-null female mice (Figure 3-3B). Baseline ACTH levels were elevated in the mutant mice compared to control, consistent with their high basal corticosterone levels (48±7 vs 29±2 pg/ml, $P<0.02$). As expected, the reduction in corticosterone stimulated a significant rise in ACTH levels in control female mice (4.6-fold over baseline), measured after six hours, and we observed a proportional significant rise in ACTH in the *Magel2*-null mice (4.2-fold over baseline, Figure 3-3C). Finally, no abnormalities were observed in the size and appearance of the adrenal or pituitary glands dissected from *Magel2*-null mice (data not shown).


A
corticosterone (ng/ml)
400
350
300
250
200
150
100
50
0
**
***
*
n.s.
baseline
dexa
baseline
dexa
baseline
dexa
baseline
dexa
control
Magel2-null
control
Magel2-null
males
females


B
corticosterone (ng/ml)
400
350
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200
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100
50
0
*
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baseline
Met.
baseline
Met.
control
Magel2-null

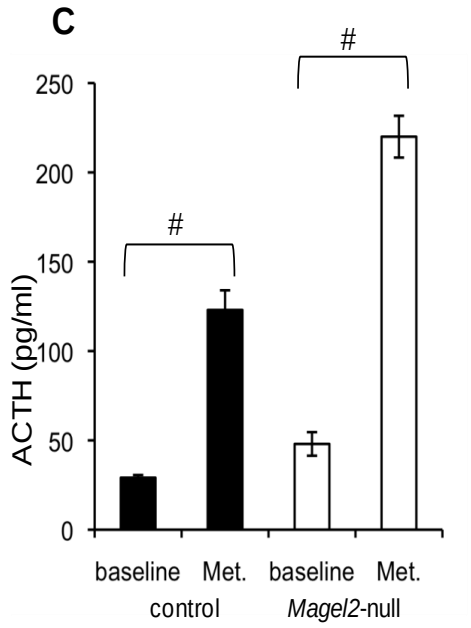
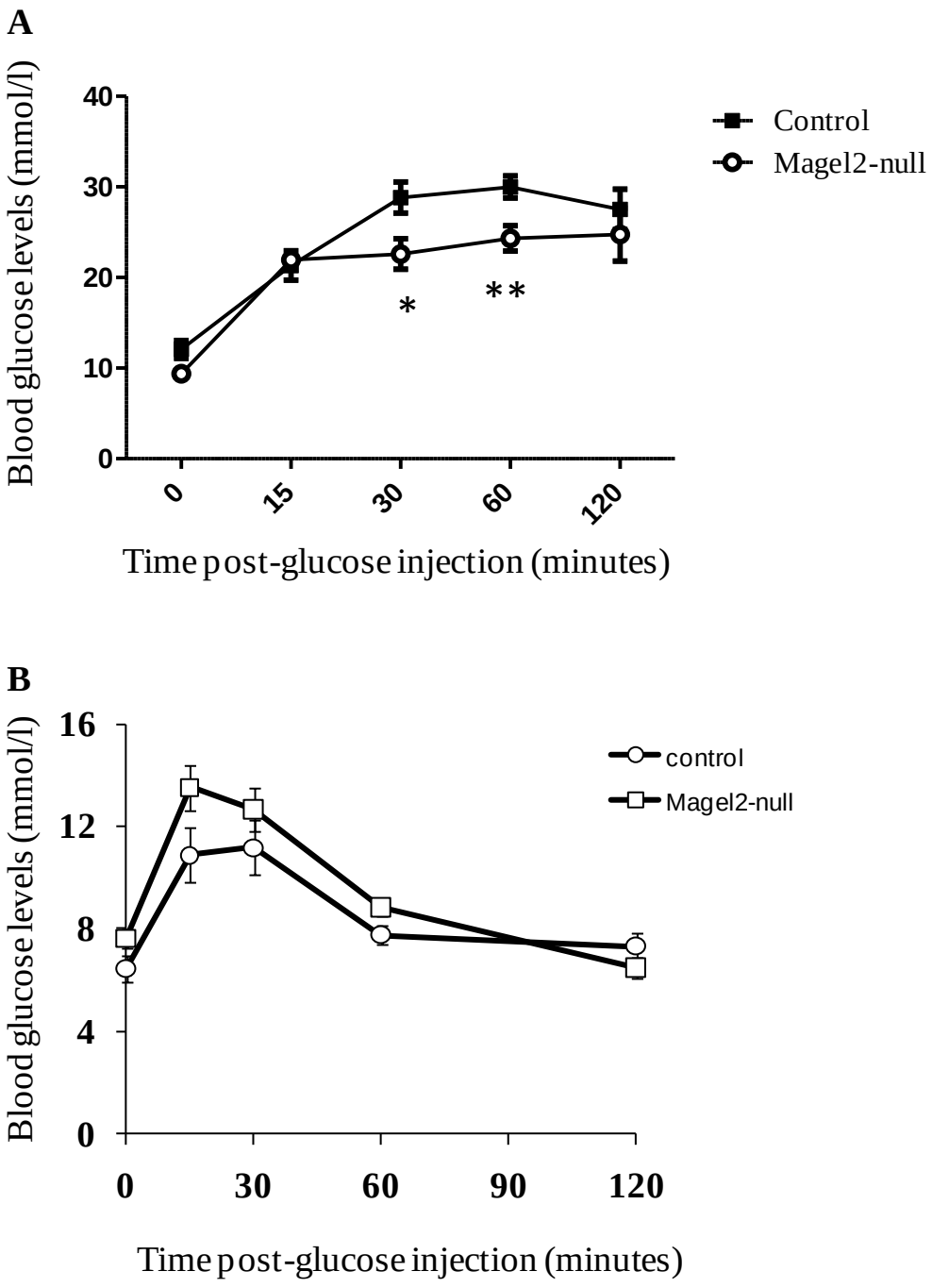

C
ACTH (pg/ml)
250
200
150
100
50
0
#
#
baseline
Met.
baseline
Met.
control
Magel2-null

Figure 3-3. Examination of the HPA axis in *Magel2*-null mice.

Values represent the mean±SEM, n=7-12 per genotype per sex. Black bars represent control, white bars represent *Magel2*-null. (A) Dexamethasone suppression of corticosterone levels. Corticosterone levels were significantly lower than baseline four hours following i.p. injection with dexamethasone (Dex), except in *Magel2*-null females. (B) Metyrapone stimulation test. Corticosterone levels were significantly lower one hour after metyrapone (Met) injection, in female mice of both genotypes. ACTH levels were measured in untreated female mice (baseline) and in female mice 6 hours after metyrapone injection (Met). ACTH levels increased significantly in both genotypes of mice (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, #, $P<0.00001$; n.s., not significant).

3.3.4 Magel2-null mice have increased insulin sensitivity

The normal decline in glucose in response to insulin suggests that *Magel2*–null mice maintain insulin sensitivity despite their increased adiposity. We further assessed insulin sensitivity in *Magel2*-null male mice compared to control littermates that were fed a high fat diet for eight weeks, and consequently developed similar adiposity as *Magel2*-null mice (obese control). At this time, HOMA-IR values (homeostasis model assessment of insulin resistance) were lower in *Magel2*-null mice than obese control (10±4 vs 19±8, $P<0.04$). *Magel2*-null and obese control mice were fasted, then administered an intraperitoneal glucose tolerance test (GTT) (McGuinness et al. 2009). In obese control mice, plasma glucose levels reached their maximum 60 minutes after glucose challenge, before beginning glucose elimination. In contrast, glucose values rose over the first 15 minutes then stabilized to lower levels in *Magel2*-null mice, indicating enhanced glucose tolerance (Figure 3-4A). A GTT was also performed in regular chow-fed mice, which allowed us to examine the ability of *Magel2*-null mice, with an innate two-fold increase in fat mass, to respond to increased blood glucose levels. The *Magel2*-null mice trended toward being more glucose intolerant than their control littermates. Interestingly, at the 120 minute time point, the blood glucose levels in *Magel2*-null mice appeared to be continuing to decrease below baseline levels (Figure 3-4B). To investigate whether altered adiponectin levels were associated with altered insulin sensitivity as they are in human populations and some rodent models of obesity, we measured total and high molecular weight adiponectin. Both total and high-molecular weight adiponectin were higher in *Magel2*-null female mice than non-obese control, but neither measurement differed between genotypes in male mice (Figure 3-4C). The ratio of high molecular weight to total adiponectin was not different from control in either sex.


A
Blood glucose levels (mmol/l)
40
30
20
10
0
0
15
30
60
120
*
**
Control
Magel2-null
Time post-glucose injection (minutes)
B
Blood glucose levels (mmol/l)
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12
8
4
0
0
30
60
90
120
control
Magel2-null
Time post-glucose injection (minutes)

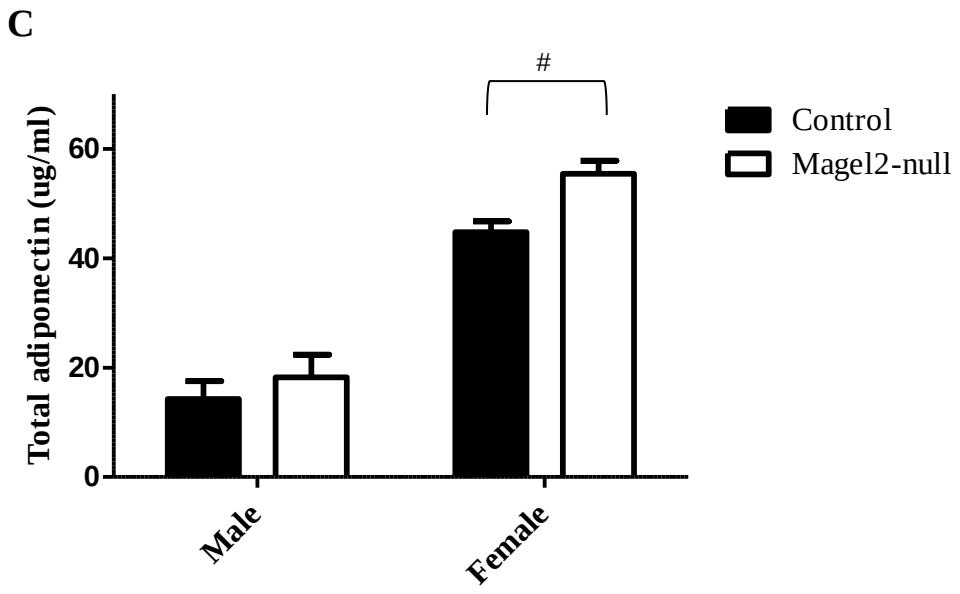

Figure 3-4. Examination of glucose tolerance and serum adipokines in *Magel2*-null mice.

(A) Glucose tolerance test (GTT) in obese control (n=7) and *Magel2*-null male mice (n=6). Values represent the mean±SEM. *Magel2*-null mice have enhanced glucose tolerance compared to obese controls. *P, <0.05; **, P<0.01. (B) GTT in control and *Magel2*-null male mice (n=8 for both genotypes). Values represent the mean±SEM. Magel2-null mice (C) Serum total adiponectin levels. Values represent the mean±SEM, (n=4-9 per genotype per sex). #*P*<0.005 when null and control results for total adiponectin are compared.

3.3.5 Growth hormone release is impaired in response to ghrelin but not GHRH in Magel2-null mice

Circulating levels of insulin-like growth factor- 1 (Igf-1) are a relatively stable indicator of long-term growth hormone (GH) secretion. While Igf-1 levels were similar in male *Magel2*-null and control littermates, female *Magel2*-null mice had 1.4-fold lower Igf-1 levels compared to control (67±4 vs 95±6 ng/ml, $P<0.002$) (Figure 3-5A). This result prompted us to investigate stimulated GH release in the female mice. We found equivalent baseline GH levels in both genotypes, although as expected from the highly pulsatile nature of GH release in rodents, there was a large variation in these levels (control 0.8±0.2 ng/ml, *Magel2*-null 0.5±0.2 ng/ml, $P>0.05$). Intraperitoneal injection of ghrelin normally elicits a robust increase in circulating GH levels in mice, by targeting growth hormone-releasing hormone (GHRH) neurons in the arcuate nucleus of the hypothalamus (Osterstock et al. 2010). Because the stimulated GH levels were not normally distributed, we used a Mann-Whitney test to evaluate whether there is a difference in the GH response of *Magel2*-null mice after ghrelin stimulation. *Magel2*-null female mice do not respond to a similar extent when compared to control female mice (control 13.8±2.9 ng/ml, *Magel2*-null 8.7±2.4 ng/ml, $P<0.05$) (Figure 3-5B). Next, we investigated the GH response to injected GHRH, which acts directly on cognate receptors in the anterior pituitary to stimulate GH release. GH response to stimulation with GHRH was similar in both genotypes (data not shown), suggesting that pituitary stimulation of GH release is normal while hypothalamic stimulation of the GH pathway is impaired in *Magel2*-null mice.

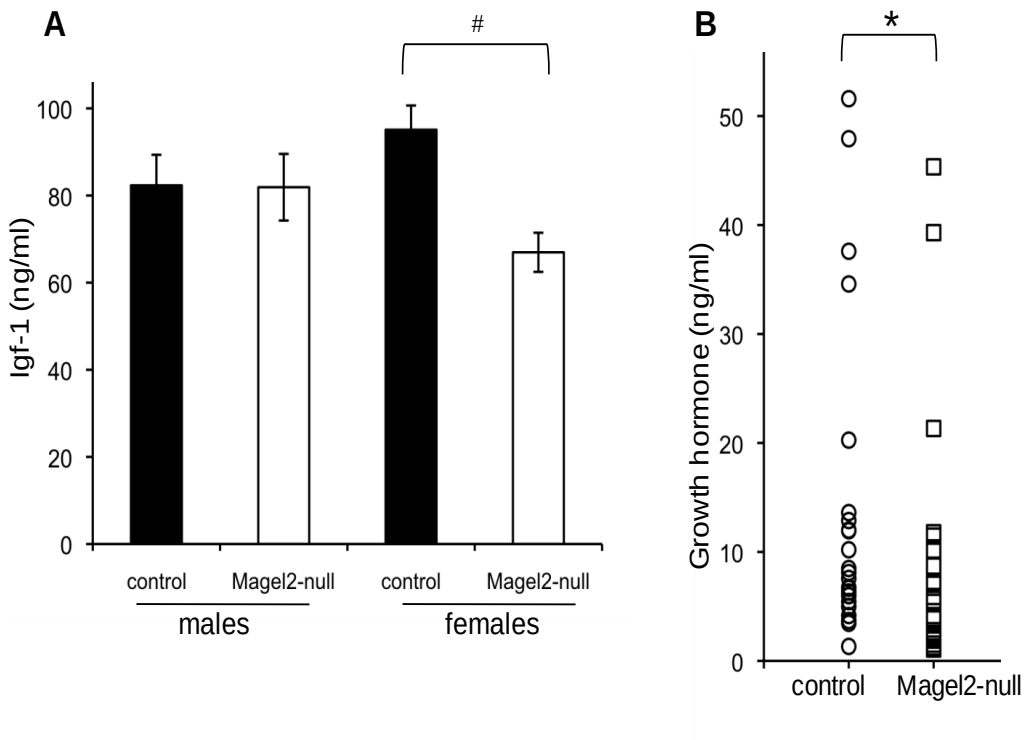

Figure 3-5. Analysis of the growth hormone pathway in *Magel2*-null mice.
(A) Serum Igf-1 levels were determined as a long-term indicator of GH axis function. Values represent the mean±SEM, n=5-8 per genotype per sex, black bars represent control, white bars represent *Magel2*-null. *Magel2*-null female mice have reduced levels of Igf-1, while *Magel2*-null male mice have similar Igf-1 levels compared to control littermates. #$P<0.01$ when null and control results are compared. (B) Stimulated serum GH levels were measured after ghrelin injection. Using a non-parametric Mann-Whitney test, the GH response to ghrelin stimulation in *Magel2*-null female mice is reduced. n=23-24 per genotype, white circles represent control, white squares represent *Magel2*-null. *$P<0.05$ when null and control results are compared.

3.3.6 Hypothalamus-pituitary-thyroid measurements

Low levels of thyroid hormones stimulate the production of hypothalamus-derived thyrotropin-releasing hormone (TRH), and pituitary-derived thyroid stimulating hormone (TSH), stimulating the production of thyroid hormones. We found that the mean T_4 levels were reduced 1.3-fold in female *Magel2*-null mice compared to control (2.9± 0.2 μg/dl compared to 3.9± 0.2 μg/dl, $P<0.002$).

3.4 Discussion

Endocrine dysfunction is a key feature of PWS, but endocrine studies in mouse models of PWS have been limited. Moreover, noteworthy differences between typical obese and PWS obese individuals have suggested that factors beyond excessive consumption versus energy expenditure mediate metabolic and endocrine dysfunction in PWS. In particular, a growth hormone deficiency associated with decreased Igf-1, increased adiposity, and decreased lean mass is partially normalized in PWS children undergoing GH replacement therapy (Eiholzer et al. 2004; Mogul et al. 2008; Carrel et al. 2010). Other endocrine abnormalities are less well defined in PWS, but include incomplete puberty, decreased stress responses, higher than expected insulin sensitivity, and possibly hypothyroidism. Many of these findings have been proposed to be hypothalamic in origin, but a molecular or genetic basis for this dysfunction has been elusive. We now show that loss of the *Magel2* gene causes abnormalities in multiple endocrine axes controlled by the hypothalamus (summarized in Table 1).

Table 3-1. Endocrine changes in Magel2-null mice.

Parameter	Female	Male
Basal corticosterone	elevated	elevated
Corticosterone response to restraint	reduced	equivalent
Corticosterone response to dexamethasone	no response	equivalent
Corticosterone response to metyrapone	normal	n.d.
Corticosterone response to hypoglycemia	no response	equivalent
ACTH	elevated	n.d.
ACTH response to metyrapone	equivalent	n.d.
Minimum glucose after insulin	reduced	n.d.
Glucose recovery rate	reduced	reduced
Insulin sensitivity	n.d.	elevated
Adiponectin	elevated	equivalent*
Igf-1	reduced	equivalent**
GH	equivalent to control	equivalent
GH response to ghrelin	fewer responsive	n.d.
T_4	reduced	n.d.

Data are summarized from a variety of experiments described in the text. Null mice are compared to chow fed sex-matched control littermates, except insulin sensitivity, where mice were compared to high fat diet fed sex-matched control littermates. n.d., not done. *high considering high adiposity, **low considering high adiposity.

3.4.1 Increased adiposity in Magel2 null mice is associated with abnormalities in the Igf-1 axis

Magel2-null mice have abnormal body composition, with fat mass 2.8 standard deviations above the mean for control mice, and lean mass 3.2 standard deviations below the control mean, and while they consume fewer calories than their wild-type littermates, their food consumption is excessive for their level of activity (Bischof et al. 2007). Diet-induced obese rodents typically have high Igf-1 levels in proportion to their increased fat mass (Roberts et al. 2010), while obese rodents with impaired GH secretion secondary to mutations in the GH pathway or leptin receptor signaling pathway (e.g. *fa*/*fa* rats) have low Igf-1 levels (Ahmad et al. 1993). We found that *Magel2* have low (female) or equivalent (male) Igf-1 levels compared to lean control, suggesting that their increased adiposity reflects GH deficiency rather than diet-induced obesity. We next measured growth hormone release in response to stimulation with ghrelin, which acts on neuronal receptors to produce GHRH and thereby stimulate release of GH from the anterior pituitary (Osterstock et al. 2010). We documented a minimal GH response in the ghrelin stimulation assay in about half the *Magel2*-null female mice, but found a normal response in the GHRH stimulation test suggesting normal pituitary function in GH synthesis and release. Overall, *Magel2*-null mice exhibit an increased fat to lean mass ratio, reduced Igf-1 levels considering their fat mass, and reduced rate of response in a growth hormone stimulation assay, all suggestive of a growth hormone deficiency of hypothalamic origin. Igf-1 levels were also recently measured in mice carrying a gene-targeted deletion of a different PWS candidate gene, *SNORD116/MBII-85* (Ding et al. 2008). In contrast to *Magel2*-null mice, these mutant mice suffer from severe postnatal growth retardation and low fat mass. Similar to other calorie restricted lean mice (Fenton et al. 2009; Imrie et al. 2009), *Snord116/Mbii-85* mice exhibit reduced serum Igf-1; growth hormone secretion was not reported in this strain.

3.4.2 Correlations among circulating adipokines, insulin sensitivity, and GH pathways in Magel2-null mice

In typical obesity, circulating levels of the adipose tissue hormones are either increased (leptin) or decreased (adiponectin), and insulin resistance is common (Saltiel 2001; Kubota et al. 2007; Fenton et al. 2009). In contrast, increased insulin sensitivity is observed in mice with mutations in the GH pathway and in people with PWS (Schuster et al. 1996). Further, adiponectin levels are higher than expected from their degree of adiposity in PWS (Hoybye et al. 2004; Haqq et al. 2007) and in severely obese adults with growth hormone receptor deficiency (Laron syndrome) (Kanety et al. 2009), suggesting a possible association between disrupted GH pathways and adiponectin. However, treatment with GH has no effect on adiponectin levels in GH-deficient children (Ciresi et al. 2007) or in adults with PWS (Hoybye et al. 2004). Thus, elevated adiponectin in PWS does not appear to be causally linked with GH deficiency or therapy. We measured adiponectin and insulin sensitivity in *Magel2*-null mice, which exhibit a two-fold increase in fat mass with a proportionate increase in leptin (Bischof et al. 2007), and which would as a result be expected to have low adiponectin. However, adiponectin levels are elevated (female) or normal (male) compared to levels in lean controls, and insulin sensitivity is normal or increased as shown in both a glucose tolerance test and insulin-induced hypoglycemia (Figure 3-2, 3-4, and Table 3-1). This phenotype is consistent with a state of growth hormone deficiency, and with low Igf-1 levels relative to adiposity in these mice.

3.4.3 Magel2 is required for normal function of the HPA axis

The hypothalamic-pituitary-adrenal (HPA) axis monitors and responds to physiological and psychological stress. Basal plasma corticosterone levels were elevated in both male and female *Magel2*-null mice, as were basal ACTH levels (Figure 3-1, Table 3-1). Elevated basal glucocorticoids are consistent with behavioral changes we previously reported in the *Magel2*-null mice, including increased baseline freezing times and reluctance to explore novel objects (Mercer et al. 2009). High basal corticosterone levels are not typical of other obese mice,

but are found in rodents with deficiencies in leptin signaling pathways (e.g. *ob/ob* mice, *db/db* mice, and *fa/fa* rats) (Saito and Bray 1983; Cohen et al. 2001; Chiba et al. 2009). The acute responses to changes in circulating corticosteroids were examined using a dexamethasone suppression test and a metyrapone stimulation test (Figure 3-3). Administration of dexamethasone induced a reduction in circulating corticosterone in male but not female *Magel2*-null mice. Inhibition of corticosterone synthesis with metyrapone induced a decrease in corticosterone, and provoked an appropriate increase in ACTH levels in both control and *Magel2*-null female mice. Together these results suggest that *Magel2*-null mice have high circulating glucocorticoid levels, but that their central responses to induced changes in these levels are comparatively normal. This result contrasts with impaired responses seen in individuals with PWS. This difference may reflect the pituitary and adrenal gland abnormalities that are observed in many individuals with PWS but that are not present in mice missing *Magel2*, and that therefore may be caused by a deficiency in a different gene inactivated in this multi-gene disorder. Importantly, both the pituitary and hypothalamus respond to changes in glucocorticoid levels, so neither test (dexamethasone or metyrapone) is specifically diagnostic for hypothalamic function.

Abnormalities in glucocorticoid mediated stress responses are a significant cause for concern during illness in people with PWS, and may have hypothalamic, pituitary, and adrenal components. We examined the central response to two types of stress in *Magel2*-null mice. Restraint stress activates the HPA axis through input from peripheral sensory information, the nucleus tractus solitaris in the brainstem, and forebrain reactive responses, integrated in the paraventricular nucleus of the hypothalamus (Herman et al. 2003; Ulrich-Lai and Herman 2009). These neurons produce hormones required for ACTH release from the anterior pituitary under both basal and stressed conditions. In contrast, hypoglycemia directly activates glucose-sensing neurons in the ventromedial hypothalamus, which initiate a counter-regulatory response, including HPA activation, to normalize blood glucose (Borg et al. 1994; Routh 2003; Tong et al. 2007). Genetic and physical lesions in the hypothalamus impair the hypoglycemia

response, even in animals that have relatively normal glucose homeostasis under fed conditions (Hochgeschwender et al. 2003; Rudic et al. 2004). Female *Magel2*-null mice exhibited a blunted response to the anticipatory stress produced by restraint, and a delayed, inadequate response to the physiological stress provoked by insulin-induced hypoglycemia (Figures 3-1, 3-2). In the latter experiment, *Magel2*-null mice became more profoundly hypoglycemic than their control littermates, and some mutant mice declined below 1.3 mmol/1 (23 mg/dl) blood glucose in the insulin tolerance test, nearing the limit for the induction of hypoglycemic shock. Notably, we only used a 2-hour fast before the insulin test rather than a more typical 6-12 hour fast, to obviate morbidity from severe hypoglycemia in the mutant mice. Thus, the defective hypoglycemia response in the *Magel2*-null mice may actually be more severe than that demonstrated in the results presented here. Further, mutant mice had a delayed onset of recovery, reduced glucose recovery rate, and in females, failure to release corticosterone and an extended time to re-attain euglycemia. Further studies are needed to determine why *Magel2*-null ventromedial hypothalamic neurons are defective in glucose-induced excitatory or inhibitory responses (Borg et al. 1994). The ventromedial hypothalamus is also considered the satiety center of the hypothalamus (Hetherington and Ranson 1942). The deficient counter-regulatory hypoglycemia response that we observe may be only one manifestation of a ventromedial hypothalamus deficiency, motivating future testing of satiety responses in these mice. In summary, our observation that both restraint stress and the counter-regulatory hypoglycemia responses are impaired in *Magel2*-null mice strongly suggests faulty integration of physiological signals in the hypothalamus in these mice, but relatively normal regulation of the HPA axis at the level of the pituitary gland and the feedback loops that regulate ACTH production.

3.4.4 MAGEL2 may be a genetic basis for defective counter-regulation of hypoglycemia in PWS

Hypersensitivity to exogenous insulin has been noted in PWS individuals, including reductions of 50% in serum glucose in almost all PWS cases but in only

a minority of obese controls (Bray et al. 1983), and severe insulin-induced hypoglycemia in earlier case reports (Sareen et al. 1975). Blunting of this counter-regulatory hypoglycemia response is a major concern for diabetics who have suffered repeated hypoglycemic episodes. Likewise, this study may motivate increased awareness of the importance of avoiding hypoglycemic episodes in individuals with PWS who are not frankly diabetic, but in whom the hypoglycemia counter-regulatory response may be congenitally impaired by loss of the *MAGEL2* gene. Monitoring for hypoglycemia is additionally imperative during fasting, for example during acute illness or before medical procedures, because co-existing impaired responses to other physiological stresses could lead to increased morbidity in this vulnerable population. In addition, therapies that target hypothalamic response to hypoglycemia and sensing of fat-derived hormones could ameliorate faulty metabolic sensing in individuals with PWS, potentially reducing their high predisposition to obesity.

In summary, *Magel2*-null mice recapitulate phenotypes of Prader-Willi syndrome with respect to body composition (Hoybye et al. 2002; Bischof et al. 2007; Mercer et al. 2009), delayed insulin-induced hypoglycemic responses, hypothyroidism, impaired growth hormone release, and, despite significantly increased adiposity, low/normal Igf-1 and high/normal adiponectin. Of note, a young woman was recently described who carries a chromosomal rearrangement that caused deletion of *MAGEL2* and two nearby genes, but left intact clusters of snoRNAs that are proposed to be important in some phenotypes of PWS (Kanber et al. 2009). This girl (their Patient 1) was investigated for PWS because of feeding problems in infancy, developmental delay, obesity with body mass index over the 97th percentile at 7 and 12 years of age, excessive eating without documented hyperphagia, and precocious puberty, but did not meet clinical criteria for PWS. Although only a single case report, this phenotype suggests that loss of *MAGEL2* contributes to PWS through effects on feeding in infancy and imbalance of food intake over energy expenditure, leading to increased adiposity and excessive weight gain in childhood.

Magel2 is most highly expressed in the nervous system, primarily in sites in the hypothalamus that include the suprachiasmatic, arcuate, and other nuclei. The phenotypes observed in mice with loss of *Magel2* are consistent with our hypothesis that Magel2 has an important role in homeostatic functions controlled by the hypothalamus. Blunted circadian rhythm (Kozlov et al. 2007) and the progressive decay in estrus cycles in female mice (Mercer and Wevrick 2009) suggest defective output from suprachiasmatic nucleus neurons. The weak, delayed response to hypoglycemia and weak response to restraint stress also point to defective integration in the ventromedial and paraventricular hypothalamic nuclei. A defective response in the arcuate nucleus likely causes the reduced GH secretion in response to ghrelin. While adiponectin has a major mode of action in muscle and liver, it also acts in the paraventricular and arcuate nuclei of the hypothalamus (Qi et al. 2004; Dridi and Taouis 2009; Hoyda and Ferguson 2010). Thus, many peripheral signals, including leptin, insulin, adiponectin, low glucose, and ghrelin converge on the hypothalamus to modulate metabolism (Cohen et al. 2001; Dridi and Taouis 2009; Hoyda and Ferguson 2010). It is tempting to speculate that a hypothalamic deficiency causes a state of altered sensitivity to peripheral hormones, thereby creating the complex phenotype that ultimately causes growth hormone-deficient obesity and other physiological dysfunction in *Magel2*-null mice.

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Chapter 4. Conclusion

4.1 Necdin is required for neuronal survival, migration, and axonal outgrowth in sympathetic neurons

When I joined the Wevrick laboratory, the emphasis of our research was examining abnormalities in the brain of *Ndn*-null embryos and identifying necdin-interacting proteins. We identified a commissural defect; and abnormal outgrowth of axons extending through the internal capsule, of central serotonergic and noradrenergic neurons, and of sympathetic neurons in cell culture (Lee et al. 2005). We also demonstrated that necdin and Magel2 interact with two Bardet-Biedl syndrome (BBS) proteins and fasciculation and elongation protein zeta (Fez1) near the centrosome. Specifically, *Ndn*-null sympathetic neurons did not survive well in culture; had less bundled axons and abnormal secondary branching of obviously shortened axons. Given necdin's established role in the differentiation of various neuronal subtypes, I hypothesized that necdin may be required for the development of sympathetic neurons. I also wanted to determine if necdin is required for the development of the entire peripheral autonomic nervous system, as a number of PWS features indicate defects in the autonomic nervous system (ANS).

Abnormal migration of sympathetic neurons in the absence of necdin

I began by examining the superior cervical ganglion (SCG) on cryosections from *Ndn*-null embryos to determine if the difficulty in culturing these neurons might be explained by an obvious *in vivo* difference. Interestingly, the SCG in the *Ndn*-null embryos were consistently located in a more caudal location and had an elongated shape (Figure 2-1). This observation potentially indicated a new role for necdin in the migration of neurons but first I had to determine if differentiation of these neurons occurs normally in the absence of necdin. I determined that specification of the sympathetic precursors was normal by examining *Phox2b* expression (Figure 2-2A), a transcription factor expressed by all noradrenergic neurons (Goridis and Rohrer 2002). This indicated that ventral migration of the vagal neural crest cells that form the SCG is unaffected by loss of necdin.

Next, I wanted to determine at what stage in development the defect is first visible in our *Ndn*-null embryos. Identifying when the defect is first observed may help elucidate which pathway or cellular process is affected in *Ndn*-null embryos. Further examination of the developing SCG identified abnormal rostral migration of the SCG neurons beginning at mid-gestation, (E12.5) that did not resolve by E18.5, just before birth (Figure 2-2B-D). Abnormal location of the SCG is also observed in transgenic mice which lack expression of *Ret* tyrosine kinase, *GDNF-family receptor alpha-3* (*Gfrα-3)*, and *artemin* (*ARTN*), all components of the same signaling cascade (Nishino et al. 1999; Enomoto et al. 2001; Honma et al. 2002). I wanted to determine if necdin may interact with the intracellular component of Ret. We determined that necdin does not interact with either the phosphorylated (active) or unphosphorylated (inactive) form of Ret in cells stably expressing Ret tyrosine kinase and transiently transfected with necdin (L. Mulligan, personal communication). Therefore, it is unlikely that loss of necdin in developing sympathetic neurons affects Ret signaling, although it has not been confirmed that downstream signaling via Ret and Gfrα family members occurs normally in the absence of necdin.

To determine if the SCG defect was present in other mouse models of PWS, I cryo-sectioned PWS-IC deletion embryos at E16.5 and examined the location of the SCG. Preliminary data indicates that the SCG defect is not 100% penetrant in the PWS-IC deletion embryos as it is in our *Ndn*-null embryos. Genotyping of PWS-IC deletion embryos by examining the location of the SCG was only accurate in half of the embryos studied (n=8 per genotype, Fisher's exact t-test, P-value = 0.08). Strain-dependent differences in the various *Ndn*-null mouse models may also explain variations in the degree of deficit observed in neuronal differentiation, migration, and axonal outgrowth.

Increased cell death and reduced axonal outgrowth in Ndn-null embryos

As the SCG in *Ndn*-null embryos at E18.5 was consistently smaller in size compared to controls (Figure 2-1), I wanted to determine if the size difference was due to increased apoptosis or reduced proliferation in the SCG throughout its

development. There was no difference in the number of proliferating cells at various developmental stages from mid-gestation to the period right before birth. I did identify an increase in cell death at E18.5 in *Ndn*-null embryos (Figure 2-3), which may be caused by necdin-deficiency or may be due to the lack of trophic factors capable of reaching the more caudally located SCG, as lack of NGF signaling would lead to reduced survival of these neurons. This observation might explain the reduced survival of SCG neurons in culture as they were potentially already dying due to the lack of adequate growth factors *in vivo*. Innervation deficits of SCG target tissues were identified in *Ndn*-null embryos (Figure 2-4) which is consistent with the axonal outgrowth defect observed *in vitro*. The vertebral nerve extending rostrally from the stellate ganglion and extensions from axons of the visceral complex are shorter in *Ndn*-null embryos (Figure 2-5B-D). These differences are consistent with axonal outgrowth deficits observed in other parts of the nervous system in *Ndn*-null embryos (Kuwako et al. 2005; Lee et al. 2005). Sympathetic innervation of other tissues may also be affected but trapping of the stain made it difficult to examine axonal projections in the liver and lungs.

Loss of necdin does not affect the development of the parasympathetic and enteric nervous systems

As loss of necdin expression affects the developing sympathetic nervous system, I wanted to determine if the development of the peripheral parasympathetic and enteric nervous systems may also be altered in the absence of necdin. To evaluate this possibility, I examined the cranial parasympathetic ganglia and determined that their location, size, and shape are preserved in *Ndn*-null embryos. To examine the developing enteric nervous system (ENS), I performed RNA *in situ* hybridization with a probe to *Ret*, a receptor expressed by all ENS neurons, in the *Ndn*-null embryo. As *Ret* expression is normal in the developing intestinal tract in *Ndn*-null embryos at mid-gestation (Figure 2-6), it is unlikely that loss of necdin expression alters the differentiation or migration of enteric neurons in the gut. Acetylcholinesterase staining of the gut did not

identify any differences in the innervations pattern of the ENS in adult *Ndn*-null mice (Figure 2-7). However, this was anticipated as *Ret* expression was normal in *Ndn*-null embryos.

Ret-null embryos have no enteric neurons in their small or large intestines however, there is bile content present in the hindgut at E18.5 and no difference in the number of bile boluses compared to control embryos (Anderson et al. 2004). Therefore, the authors concluded that the ENS is not required for propulsion of gut contents or the presence of slow waves, which only appear postnatally. Preliminary examination of the GI tract in E18.5 *Ndn*-null embryos identified decreased transit of bile along the colon in *Ndn*-null embryos compared to controls. Decreased fetal propulsion of gut contents in *Ndn*-null embryos implies that the decreased GI motility in PWS is caused by deficits in smooth muscle function versus a deficit in the ENS. Measuring spontaneous contractile activity of the small intestine in *Ndn*-null mice may identify a defect in motility along the gut and may aid in determining the location of the defect. Although loss of necdin appears to affect the development of the sympathetic nervous system, it does not adversely alter the development of neurons in the other peripheral autonomic systems.

4.2 Loss of necdin causes defects in the development and function of the nervous system

To date, necdin is the most studied protein in the Prader-Willi syndrome (PWS) region. Necdin was originally identified in a screen of neurally differentiated embryonal carcinoma cells (Maruyama et al. 1991) and was found to be expressed in mouse brain (Aizawa et al. 1992). Further *in vitro* studies identified a role for necdin in growth suppression (Taniura et al. 1998; Taniura et al. 1999); and in terminal differentiation of neurons as PC12 cells transfected with necdin have increased differentiation, while repression of necdin in embryonic dorsal root ganglia (DRG) suppresses their differentiation (Takazaki et al. 2002; Tcherpakov et al. 2002). Necdin has also been identified as a key mediator of

neurite outgrowth as expression of necdin in PC12 and neuroblastoma cells both induces and accelerates neurite outgrowth (Kobayashi et al. 2002). Necdin is expressed throughout the mouse embryonic nervous system with the highest expression in the hindbrain and hypothalamus (Lee et al. 2003), and correlating with neuronal differentiation (Andrieu et al. 2003).

The data I present in this thesis is consistent with *in vitro* studies of necdin and with observations in multiple transgenic mouse models who are functionally *Ndn*-null. While studying the sympathetic deficit in our *Ndn*-null embryos, a study on the fourth *Ndn*-null mouse model was published, in which the authors identified the interaction of necdin with tropomysin-regulated kinase A (TrkA), the high affinity receptor for NGF, in peripheral sensory neurons, specifically, embryonic DRGs (Kuwako et al. 2005). Reduced activation of downstream targets, including a decrease in phosphorylated ERK1/2, members of the MAP kinase family, were observed in *Ndn*-null DRG. Studies in *TrkA*-/- mice identified increased cell death in the SCG perinatally, while in *TrkC*-/- mice, no SCG deficit is observed, indicating a specific role for TrkA in SCG development (Huang and Reichardt 2001). As necdin interacts with TrkA, loss of this interaction may also lead to reduced survival of sympathetic neurons and abnormal branching of the already shorter axons. A similar study described increased apoptosis in *Ndn*-null DRGs at a developmental stage when programmed cell death is naturally occurring (Andrieu et al. 2006). The authors excluded an anti-proliferative role for necdin in early development of DRG and determined it likely functions as a survival factor, as well as being essential for neuronal differentiation. Previous studies identified an interaction between various MAGE proteins, including necdin, and $p75^{NTR}$, the low affinity neurotrophin receptor (Tcherpakov et al. 2002). In *Ndn*-null DRG, the survival of $p75^{NTR}$-expressing neurons is not compromised, although, it does not rule out the possibility that altered signaling occurs in these neurons. Therefore, the additional loss of an interaction between necdin and $p75^{NTR}$ might potentially affect signaling in the *Ndn*-null SCG and cause migration, survival, and axonal outgrowth defects.

Further characterization of necdin in cellular migration was performed in mouse embryonic fibroblasts (MEFs), limb bud cultures, and neuronal cultures from *Ndn*-null embryos (Bush and Wevrick 2010). Polarization in a wound-healing assay of *Ndn*-null MEFs and PWS patient fibroblasts is delayed compared to control samples and is caused by impaired myosin-dependent activities. These results indicate that SCG migration defects are likely caused by a failure of the neurons to polarize in response to NGF and NT-3 through their activation of TrkA. Tangential migration of GABAergic forebrain neurons is also decreased in *Ndn*-null embryos (Kuwajima et al. 2010) and the loss of GnRH neurons may be caused by reduced migration in addition to abnormal differentiation of the precursor cells (Miller et al. 2009). How loss of necdin affects axonal outgrowth is not currently known however, cultured *Ndn*-null hippocampal neurons fail to extend their axons in the direction of an exogenous source of BDNF (Bush and Wevrick 2010), indicating that loss of necdin appears to affect TrkB signaling. This is further supported by *in vitro* cultures of *Ndn*-null embryonic DRG, in which reduced neurite extension is observed in the presence of NGF (Kuwako et al. 2005). It is likely that altered Trk signaling in PWS is responsible for abnormalities observed in brain images (Miller et al. 2007; Miller et al. 2007) by causing reduced axonal outgrowth and increased apoptosis, and contributing to global developmental delay and psychiatric illnesses.

As the ENS appears normal in the absence of necdin, the gastrointestinal (GI) dysmotility observed in PWS individuals may be caused by defects in relaying information from the gut to the autonomic centres in the hypothalamus and/or brainstem (Figure 1-5). Previous studies have identified defects in the development of nuclei in the brainstem of our *Ndn*-null embryos, including the nucleus tractus solitaris (Pagliardini et al. 2005), a key neuronal integration centre. Reduced differentiation of smooth muscle cells in the absence of necdin (Brunelli et al. 2004) may also be the cause of reduced GI motility as the hypotonia observed throughout the body may also persist along the length of the gut. Further studies in *Ndn*-null mice have identified decreased muscle regeneration due to reduced differentiation and survival of myoblasts (Deponti et

al. 2007) and altered migration of cultured limb bud cells resulting in a reduction of the size of the dorsal muscle cell progenitor pool (Bush and Wevrick 2010). These studies support a role for necdin in the development of skeletal and smooth muscle and in the pathogenesis of reduced GI motility.

4.3 Loss of Magel2 causes defects in multiple hypothalamic-pituitary axes

No candidate gene has yet been identified as the cause of hypothalamic deficits in PWS. Therefore, I wanted to determine if *NDN* or *MAGEL2* may be responsible for deficits in any of the hypothalamic-pituitary axes, as both genes are highly expressed in the hypothalamus. *Ndn*-null mice have reduced numbers of GnRH and oxytocin expressing neurons in the hypothalamus (Muscatelli et al. 2000; Miller et al. 2009). Oxytocin-null mice have altered responses to stress and develop age-dependent obesity (Amico et al. 2008; Camerino 2009). As loss of oxytocin can alter the stress response, I wanted to determine if hypothalamic-pituitary deficits may be present in our *Ndn*-null mice. I did not identify any differences in growth hormone secretion, stress responses or thyroid hormone levels in *Ndn*-null mice, however various deficits of these hypothalamic-pituitary axes were observed in *Magel2*-null mice.

Growth hormone deficiency in Magel2-null mice

Growth hormone (GH) secretion is pulsatile and difficult to accurately measure, while insulin-like growth factor 1 (IGF-1) is a surrogate marker representing the long-term activity and integrity of the GH axis (Figure 1-8). By measuring Igf-1 levels, I identified no difference in *Magel2*-null male mice but there was a decrease in Igf-1 levels in *Magel2*-null female mice (Figure 3-5A). The sex difference in Igf-1 levels was interesting as there is no sex discrimination in PWS with respect to GH deficiency and reduced IGF-1 levels (Burman et al. 2001). The most likely explanation is that species differences may affect how loss of *MAGEL2/Magel2* alters the hypothalamic-pituitary-growth hormone axis. In typical obesity, IGF-1 levels tend to be increased while GH levels are reduced

(Hoybye 2004), thus the increased adiposity and reduced or equivalent Igf-1 levels in *Magel2*-null mice is consistent with a GH-deficient phenotype.

Ghrelin stimulation of GH release has been studied in various transgenic mouse models including GH secretagogue receptor (*Ghsr*)-null mice and in transgenic obese mice (Sun et al. 2004; Iwakura et al. 2007). Therefore, I measured the GH response to ghrelin in *Magel2*-null female mice and determined that while control mice responded accordingly, approximately half of the *Magel2*-null mice did not adequately respond to ghrelin stimulation (Figure 3-5B). I also measured the GH response to GHRH stimulation of somatotropes in the pituitary and determined that *Magel2*-null mice respond similarly to their control littermates, indicating that the pituitary reserve of GH is sufficient in *Magel2*-null female mice. Reduced response to ghrelin but not GHRH stimulation indicates that the GH release deficit in *Magel2*-null mice is likely caused by a hypothalamic defect. Ghrelin stimulates GH secretion by inducing GHRH production and secretion in the arcuate nucleus. Projections from the arcuate nucleus are known to reach other hypothalamic centres involved in regulating satiety (Bagnol et al., 1999; Xu et al., 2003). This indicates another potential location in the hypothalamus of *Magel2*-null mice that is defective but requires further investigation to determine the extent of the deficiency, particularly with respect to growth hormone secretion and satiety responses.

Altered hypothalamic-pituitary-adrenal (HPA) axis responses in Magel2-null mice

Increased glucocorticoid levels were identified in *Magel2*-null mice of both sexes (Figure 3-1) and increased basal ACTH levels were observed in *Magel2*-null female mice (Figure 3-3B), indicating abnormal feedback of the HPA axis in the absence of Magel2. Male mice of both genotypes responded adequately to both restraint stress and insulin-induced hypoglycemia. However, a reduced response to stress was identified in *Magel2*-null female mice as they did not show a rise in corticosterone levels in response to insulin-induced hypoglycemia and also showed a diminished rise in corticosterone levels

following restraint (Figure 3-1). The response to dexamethasone is absent in *Magel2*-null female mice while metyrapone stimulation induces an appropriate increase in ACTH levels (Figure 3-3A&B). Male *Magel2*-null mice respond normally to dexamethasone suppression of corticosterone levels (Figure 3-3A). As sex-specific differences are common in rodents, the normal response in the *Magel2*-null male mice is not unusual. Unfortunately, the suppression and stimulation tests assess the integrity of both the hypothalamus and pituitary gland and as a result, we were not able to determine the extent of the hypothalamic deficit. Interestingly, *Magel2*-null female mice appeared to respond normally to decreased glucocorticoid levels but did not reduce corticosterone levels during a suppression test, which may explain the elevated basal levels. Measuring corticotrophin-releasing hormone (CRH*)* levels may be useful in determining the extent of the hypothalamic deficit as we would anticipate reduced levels if the feedback loop is intact, however this is not likely as basal ACTH levels remain elevated. *Magel2*-null mice are partially capable of responding to stressors, hence it is more likely that loss of proper integration between hypothalamic nuclei versus a specific loss of *Crh*-expressing neurons occurs in the absence of Magel2.

Delayed counter-regulatory response to hypoglycemia in Magel2-null mice

Glucose-sensing neurons in the ventromedial nucleus of the hypothalamus are activated by hypoglycemia and respond by inducing glucagon and glucocorticoid release to normalize blood sugar levels (Routh 2003). Lesions in the hypothalamus impair the hypoglycemia response even in animals that have normal glucose homeostasis under fed conditions (Hochgeschwender et al. 2003; Rudic et al. 2004). *Magel2*-null mice became more profoundly hypoglycemic when injected with insulin (Figure 3-2) and *Magel2*-null female mice do not recover their blood glucose levels as quickly as control mice. It might be necessary to measure glucagon levels post-insulin injection to determine if this aspect of the counter-regulatory response is normal in *Magel2*-null mice. Further studies are needed to determine why *Magel2*-null neurons in the ventromedial

nucleus are defective in glucose-induced excitatory or inhibitory responses (Borg et al. 1994) by performing electrophysiological readings of neurons in this nucleus when treated with changing glucose concentrations. The ventromedial nucleus is also considered an important satiety center of the hypothalamus along with the arcuate nucleus (Hetherington and Ranson 1942). The deficient counter-regulatory hypoglycemia response that we observe may be only one manifestation of a deficiency in the ventromedial nucleus, motivating future testing of satiety responses in these mice by examining levels of orexigenic and anorexigenic peptides post-fast and refeed.

Loss of Magel2 causes a reduction in thyroid hormone levels

Examination of the hypothalamic-pituitary-thyroid axis identified a 1.3-fold reduction of total T_4 levels in *Magel2*-null female mice. To further evaluate the hypothalamic-pituitary-thyroid axis in *Magel2*-null mice, it will be necessary to measure T_3 and TSH levels to determine if primary or central hypothyroidism is the cause of reduced T_4 levels. As Magel2 is not expressed at an appreciable level in the thyroid gland, altered thyroxine levels are likely caused by a central disruption of the hypothalamic-pituitary-thyroid axis at the level of the hypothalamus. Central hypothyroidism has recently been identified in 19-24% of PWS children (Miller et al. 2008; Diene et al. 2010) while 72% of PWS infants have free or total T_4 levels two standard deviations below those of a standard infant population (Vaiani et al. 2010). This is an important finding as infancy is a critical period for neurological developmental and reduced thyroid hormone may adversely affect this process, in addition to the deficit caused by loss of expression of PWS candidate genes in the brain. Loss of Magel2 in PWS likely contributes to hypothyroidism, particularly in infancy, which persists through childhood in some individuals.

Loss of Magel2 causes increased insulin sensitivity and an altered adipokine profile

Adiponectin is a hormone secreted by adipose tissue and its circulating levels, particularly the high molecular weight from, are reduced in typical obesity. Total and high molecular weight adiponectin levels are increased in *Magel2*-null female mice or equivalent in *Magel2*-null male mice (Figure 3-4B), which was unexpected considering their two-fold increase in fat mass. However, this increase in adiponectin despite increased adiposity is similar to what is observed in Laron syndrome individuals, who are GH insensitive, (Kanety et al. 2009) and PWS individuals who are GH deficient (Hoybye et al. 2004; Kennedy et al. 2006; Haqq et al. 2007). *Magel2*-null mice do not adequately respond to insulin injection and also have increased glucose tolerance when fed a high-fat diet (Figure 3-4A), as their blood glucose levels remain lower than those observed in control obese mice. Similar to the higher adiponectin levels, the increased insulin sensitivity is unexpected considering their increased adiposity but is consistent with a GH-deficient phenotype.

4.4 Putative role for MAGEL2 in hypothalamic development and function

Relative GH deficiency in PWS contributes to abnormal body composition consisting of increased body fat mass and reduced lean mass, and treatment with GH partially normalizes body composition (Eiholzer et al. 2004; Mogul et al. 2008; Carrel et al. 2010). Children with PWS typically have decreased spontaneous GH secretion, and low peak GH response to stimulation tests (Theodoridis et al. 1971; Burman et al. 2001), accompanied by reduced serum IGF-1 and low IGF-binding protein 3 (Eiholzer et al. 1998; Miller et al. 2008; Sode-Carlsen et al. 2010). Reduced Igf-1 levels, reduced GH stimulation in response to ghrelin, and elevated adiponectin levels support a role for Magel2 in the growth hormone deficiency observed in PWS individuals. Adiposity in *Magel2*-null mice is two standard deviations above that observed in weight-matched controls (Bischof et al. 2007), indicating a GH deficient body composition similar to that observed in PWS individuals. *MAGEL2* is also highly

expressed in the pituitary, which may be one explanation as to why the GH stimulation deficit is greater in humans than in mice. *Snord116*-deletion mice have postnatal growth retardation and remain smaller in size throughout adulthood when compared to control littermates (Ding et al. 2008). Therefore, it is also possible that the combined loss of *SNORD116* and *MAGEL2* in PWS may increase the degree of GH-deficiency observed in people compared to either single-deletion mouse model.

Fasting ghrelin levels are higher in PWS individuals and this increase precedes the onset of obesity in PWS children (Cummings et al. 2002; Haqq et al. 2003; Erdie-Lalena et al. 2006; Feigerlova et al. 2008). Circulating ghrelin levels are lower in *Magel2*-null mice (Kozlov et al. 2007) but increased in *Snord116*-null mice (Ding et al. 2008). PWS individuals gain more weight with fewer calories because of reduced activity and persistent hypotonia. Likewise, *Magel2*-null mice consume fewer calories compared to control littermates but gain more weight due to reduced activity levels (Kozlov et al. 2007). A recent study identified a role for ghrelin in maintaining blood glucose levels during severe calorie restriction (Zhao et al. 2010). If the hypothalamus in PWS individuals does not accurately integrate peripheral signals, their brain may signal that they are in a lower energy state and cause the excessive release of ghrelin in a normal state of feeding. Following exogenous insulin administration, the more profound hypoglycemia observed in PWS individuals with congenital loss of *MAGEL2* and in *Magel2*-null mice may be due to inadequate or delayed ghrelin signaling.

Identification of high basal glucocorticoid levels in *Magel2*-null mice was unexpected. However, high basal corticosterone levels are also observed in rodents with deficient leptin signaling, including *ob/ob* and *db/db* mice; and *fa/fa* rats, but are not typical of other obese mice (Saito and Bray 1983; Cohen et al. 2001; Chiba et al. 2009). The increase in basal corticosterone levels in *Magel2*-null mice may also be caused by defective leptin signaling as they have hyperleptinemia consistent with their increased fat mass. Response to peripheral leptin in newly weaned *Magel2*-null mice would have to be determined in order to

conclude that *Magel2*-null mice have a true leptin-deficient-like phenotype. Although basal glucocorticoid levels are normal in children with PWS, central adrenal insufficiency has been identified due to an inadequate ACTH response to metyrapone testing, an inhibitor of cortisol production (de Lind van Wijngaarden et al. 2008). *Magel2*-null mice appear to respond appropriately to metyrapone stimulation of ACTH secretion, indicating that while the stress-response phenotype is similar between mice and humans, it is not identical due to possible differences in protein function between species.

MAGEL2 expression follows a circadian pattern and Magel2 co-immunoprecipitates with circadian rhythm proteins, period circadian protein homolog 2 (Per2) and brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1 (Bmal1) in transiently transfected cell lines (S. Weselake, personal communication). Expression of circadian genes is regulated by glucocorticoids, specifically the promoter region of *Per2* contains glucocorticoid responsive elements (GREs) (So et al. 2009). *Per2*-mutant mice chronically treated with glucocorticoids do not develop glucose intolerance and remain insulin sensitive compared to control animals. Like *Magel2*-null mice, the *Per2*-mutant mice have increased leptin levels. *Bmal1* and *Clock*, two other circadian genes, are also involved in glucose homeostasis by regulating recovery from hypoglycemia (Rudic et al. 2004). In contrast to *Magel2*-null mice, *Bmal1*-null and *Clock*-mutant mice exhibit a normal counter-regulatory response but impaired gluconeogenesis in the liver. Increased corticosterone levels in *Magel2*-null mice may be an attempt to maintain appropriate levels of circadian rhythm proteins. This data further supports a role for Magel2 in the regulation of circadian rhythm that maintains secretion profiles of hypothalamic and pituitary hormones and in the maintenance of glucose homeostasis, though the exact mechanism remains unknown.

Although less is known about the cellular role of Magel2 in comparison to necdin, a recent study of *Maged1*-null mice identified a circadian rhythm defect similar to the one observed in *Magel2*-null mice (Wang et al. 2010). MAGED1 is

not in the PWS region but it does interact with necdin and is also expressed throughout the nervous system (Kuwajima et al. 2004). MAGED1 coordinates necdin's interaction with Dlx and Msx transcription factors and induces specification and differentiation of GABAergic neurons by facilitating necdin's binding to Dlx2 and Dlx5 (Kuwajima et al. 2006). Perhaps, Magel2 interacts with MAGED1 in the suprachiasmatic nucleus, coordinates circadian rhythm by maintaining the expression of circadian-regulated transcripts, and consequently regulates glucose homeostasis. Multiple MAGE proteins interact with $p75^{NTR}$ (Tcherpakov et al. 2002) thus it is also possible that loss of Magel2 alters neurotrophin signaling in the hypothalamus. Altered signaling within and between various hypothalamic nuclei may also explain the multiple deficits observed in *Magel2*-null mice and PWS individuals who lack both necdin and Magel2.

4.5 Hypothalamic dysfunction in other obesity syndromes and their connection to PWS

As necdin and Magel2 interact with two of the Bardet-Biedl syndrome (BBS) proteins, BBS4 and BBS8, it is possible that the cause of obesity in both disorders may be of a similar origin. BBS is a heterogeneous genetic disorder with fourteen genes known to be involved in its etiology and is caused by abnormal function of cilia in various tissues (Waters and Beales 2009). Other similarities between BBS and PWS include hypogonadism and cognitive impairment. *Bbs2*, *Bbs4*, and *Bbs6* single-deletion transgenic mouse models are hyperphagic, have reduced locomotor activity, increased leptin levels, and reduced hypothalamic pro-opiomelanocortin (POMC) expression, an anorexigenic peptide (Rahmouni et al. 2008). *Magel2*-null mice are also hyperleptinemic, implying that leptin resistance may be a common cause of obesity in these two monogenic obesity syndromes. Current studies in the Wevrick laboratory indicate that *Magel2*-null mice do not adequately decrease their food intake in response to peripheral leptin injection and are essentially leptin-resistant, even at a younger age (6 weeks) before increased adiposity has developed in these mice. The cause

of the leptin resistance appears to be an inappropriate inhibition of POMC neurons when leptin is exogenously applied to hypothalamic slice cultures (R. Mercer, personal communication). *Bbs1* and *Bbs4* knockout mice both exhibit altered peripheral thermo- and mechano-sensory innervation and function (Tan et al. 2007), which is comparable to the sensory deficits observed in *Ndn*-null mice. These similarities increase the possibility that PWS and BBS candidate genes function together in multiple cellular processes.

Recently, a study identified lower levels of serum and plasma BDNF in PWS individuals, which is thought to reflect central production levels of BDNF (Han et al. 2010). Patients who are haploinsufficient for BDNF display hyperphagia leading to severe obesity, have cognitive impairment, and are hyperactive (Gray et al. 2006). Male mice with heterozygous loss of *Bdnf* are chronically hyperphagic leading to obesity and exhibit intermale aggression, which is thought to be due to serotonin dysfunction (Lyons et al. 1999). Interestingly, the obese phenotype in the *Bdnf* +/- mice can be reversed when BDNF or NT4/5 is intracerebroventricularly infused indicating that BDNF is not required developmentally to establish the proper circuitry in food intake or energy balance pathways (Kernie et al. 2000). It would be interesting to measure serum or plasma levels of BDNF in mouse models of PWS to determine if these levels are altered, even in the absence of overt obesity as is observed in *Bdnf* +/- mice. If BDNF serum levels are altered in any of the mouse models, injection of BDNF through various routes can be studied to determine if this may be a viable treatment for hyperphagia in PWS individuals. Cultured *Ndn*-null neurons are delayed in their response to BDNF, as neurite extension is reduced compared to that observed from control neurons (Bush and Wevrick 2010). Therefore, another possibility is that loss of necdin in PWS leads to a reduced response to BDNF, thus contributing to excessive hyperphagia. As observed in *Bdnf* +/- mice, disruption of serotonergic circuits is present in the hindbrain of *Ndn*-null mice (Zanella et al. 2008) which indicates another similarity between the BDNF haploinsufficiency and PWS phenotypes.

Expression of single-minded homolog 1 (*Sim1)*, a basic helix-loop-helix transcription factor, can be first identified at E10.5 in mice and maintains its expression well into the postnatal period (Michaud et al. 1998; Caqueret et al. 2006). *Sim1*-/- mice die shortly after birth and display significant hypoplasia throughout the hypothalamus, including complete loss of the supraoptic and paraventricular nuclei; and all TRH and CRH expressing neurons. When *Sim1*'s binding partner, *Arnt2*, is deleted in mice, a similar loss of hypothalamic neuronal populations is observed, indicating that these proteins function cooperatively in the patterning of the developing anterior hypothalamus (Hosoya et al. 2001; Keith et al. 2001). As *Sim1*-/- mice die at birth, studies have relied on *Sim1*+/- mice to examine the function of Sim1 during development. *Sim1*+/- mice exhibit excessive hyperphagia, hyperinsulinemia, and increased leptin levels (Michaud et al. 2001), which overlaps with aspects of both the PWS and *Magel2*-null phenotypes. As necdin interacts with ARNT2, a next step in determining necdin's role in this pathway would be to examine brain-2 (Brn2) expression in the hypothalamus of *Ndn*-null mice, as this is a target of SIM1/ARNT2, and loss of Brn2 results in similar hypothalamic nuclei deficits (Szarek et al. 2010). *Sim1*+/- mice have moderately reduced hypothalamic expression of Crh, Avp, Trh, and somatostatin, and almost complete loss of oxytocin expression (20% of control) (Kublaoui et al. 2008). The oxytocin-deficiency is speculated to be the cause of hyperphagia observed in *Sim1*+/- mice as they do not increase hypothalamic oxytocin levels in response to fasting. A reduced number of oxytocin-expressing neurons were identified in one of the *Ndn*-null mouse models (Muscatelli et al. 2000). Also, a food intake study in our hybrid strain of *Ndn*-null mice identified increased feeding during the day when the mice should have reduced activity levels. Examining expression of hypophysiotrophic factors may also aid in elucidating a role for necdin and Magel2 in the development of hypothalamic nuclei.

4.6 Dissecting the PWS phenotype: specific roles of candidate genes in PWS

Although recent reports identified three patients with isolated deletions of the *SNORD116* snoRNA cluster, and major features of PWS, studies in cell lines and mouse models indicate a role for necdin and Magel2 in development and function of the nervous system. The contribution of various candidate genes to multiple PWS features is summarized below (Table 4-1). I propose that necdin is responsible for breathing deficits observed in PWS (Ren et al. 2003; Zanella et al. 2008; Zanella et al. 2008; Zanella et al. 2009) however loss of *SNORD116* may play a role in sleep apneas, as at least one individual with the isolated deletion of *SNORD116* has central sleep apnea (Sahoo et al. 2008). Sensory deficits are also due to loss of necdin expression (Kuwako et al. 2005; Kanber et al. 2009) and it is likely that necdin plays a larger role in regulating the autonomic nervous system than is currently known. Studies in *Magel2*-null mice indicate a role for this MAGE protein in hypothalamic-pituitary-endocrine axes including: reproduction, growth hormone deficiency, obesity, central adrenal insufficiency, and hypothyroidism (Bischof et al. 2007; Mercer and Wevrick 2009; Tennese and Wevrick 2010). Sleep disorders in PWS are likely caused by loss of *MAGEL2* as *Magel2*-null mice have a blunted circadian rhythm and a reduced number of orexin-expressing neurons, which are implicated in the regulation of sleep (Lee et al. 2005). Although no difference in the number of hypothalamic orexin neurons was observed in post-mortem brains of PWS individuals (Fronczek et al. 2005), multiple groups have identified reduced levels of orexin in the cerebrospinal fluid of people with PWS (Mignot et al. 2002; Arii et al. 2004; Nevsimalova et al. 2005). Although little is known about the function of *SNORD116*, based on studies in mice and humans, it appears to play a role in the major criteria of PWS. Hypotonia is likely caused by loss of both *SNORD116* and *NDN*, however loss of *MAGEL2* might contribute to the phenotype by altering body composition. Loss of these three genes likely also contributes to hyperphagia in PWS based on reports in patients with atypical deletions and in studies of multiple mouse models of PWS. Growth hormone (GH) deficiency, which remains a key feature of PWS, is likely caused by loss of a combination of *SNORD116* and *MAGEL2*, as

Magel2-null mice exhibit the altered body composition of GH deficiency (Bischof et al. 2007)while *Snord116*-null mice are reduced in size (Ding et al. 2008). *SNORD115* plays a role in serotonin 2C receptor pathways and causes alterations in behaviour reminiscent of losses in serotonin signaling. Global developmental delay and typical behavioural characteristics are likely caused by the loss of a combination of PWS candidate genes as they are complex traits with probable multigenic origin and environmental influences.

Table 4-1. Model for the contribution of individual genes to Prader-Willi syndrome (PWS).

PWS features	Candidate gene contribution
Reduced fetal movements, neonatal hypotonia, poor weight gain in infancy	SNRPN/snoRNA cluster[1,2], *MAGEL*[3]*2* (poor weight gain), *NDN*[4] (poor muscle formation)
Hypogenitalism,/hypogonadism/ Cryptorchidism, infertility	*SNORD116*[1,2], *NDN*[5] (fewer GnRH neurons), *MAGEL2*[6] (impaired luteinizing hormone surge)
Low birth weight	*NDN*[7]
Hyperphagia	*SNORD116*[1,2]
Childhood onset obesity, metabolic imbalance	*NDN* [4] (poor muscle formation, poor carbohydrate metabolism), *MAGEL2*[3,7] (increased fat and reduced muscle)
Sleep disturbance, excessive daytime sleepiness	*MAGEL2*[8] (circadian disruption)
Central sleep apnea, reduced response to hypoxia	*NDN*[9], *SNORD116*[1]
Autonomic nervous system deficiency including: abnormal thermoregulation, gastrointestinal dysmotility	*NDN*[10] , but full understanding of specific genes causing deficits not currently known
High pain threshold, decreased vomiting	*NDN*[7,11]
Short stature, facial features, small hands and feet	*MAGEL2*[12], *SNORD116*[1], but partially resolves with GH treatment, so likely secondary to reduced muscle tone and relative GH deficiency
Delayed milestones and mental deficiency	*SNORD116*[1], *NDN*[13], combined loss of several genes important in the development of the nervous system
Addictive and obsessive compulsive behaviour, skin picking	*SNORD116*[1,2], *MAGEL2*[14], *SNORD115*[15], and combined loss of several genes important in the development of the nervous system
Central adrenal insufficiency, hypothyroidism	*MAGEL2*[12]

1. human (Sahoo et al. 2008; de Smith et al. 2009; Duker et al. 2010)
2. mouse (Skryabin et al. 2007; Ding et al. 2008)
3. mouse (Bischof et al. 2007)
4. mouse (Kuwajima et al. 2004; Deponti et al. 2007; Bush and Wevrick 2008; Bush and Wevrick 2010)
5. mouse (Muscatelli et al. 2000; Miller et al. 2009)
6. mouse(Mercer and Wevrick 2009)
7. human (Kanber et al. 2009)
8. mouse (Kozlov et al. 2007)
9. mouse, human (Ren et al. 2003; Pagliardini et al. 2005; Zanella et al. 2008; Zanella et al. 2008; Zanella et al. 2009)
10. mouse (Tennese et al. 2008)
11. mouse (Kuwako et al. 2005)
12. mouse (Tennese and Wevrick 2010)
13. mouse (Lee et al. 2005; Kuwajima et al. 2006; Kuwajima et al. 2010)
14. mouse (Mercer et al. 2009)
15. human, mouse (Kishore and Stamm 2006; Doe et al. 2009; Kishore et al. 2010)

4.7 Future Directions

Although insight into the roles of necdin and Magel2 in the pathogenesis of PWS has been described in this thesis, future studies of their function in the nervous system will be discussed below. Further study of the central aspects of the autonomic nervous system (ANS) should be performed in *Ndn*-null mice, as the lack of parasympathetic peripheral deficits does not exclude a role for necdin in the autonomic deficits observed in PWS. Examination of the central portion of the ANS can be accomplished using neuronal tracers to identify connections between various locations in the brain. Specifically anterograde tracing of the vagal nerve can be performed by placing wheat-germ agglutinin conjugated to horse radish peroxidase in the nodose ganglion and examining the stomach and duodenum in *Ndn*-null mice to determine if axons extend similarly within their gut compared to control mice. Neural connections between the medulla and the hypothalamus are required for proper vagal motor reflexes and neurons in the medulla may provide feedback cues to the hypothalamus regarding the activity of vagal motor neurons (Hardy 1995). Placing retrograde tracers, such as DiI in hypothalamic nuclei, and the nucleus tractus solitaris and dorsal motor nucleus (cranial nerve X) in the brainstem; and examining projections between these regions would identify any connection deficits in the brains of *Ndn*-null mice.

Due to necdin's role in smooth and skeletal muscle development and function (Brunelli et al. 2004; Kuwajima et al. 2004; Deponti et al. 2007; Bush and Wevrick 2008), I propose that loss of necdin may also affect smooth muscle function in the gastrointestinal (GI) tract. When compared to lean and obese controls, gastric emptying in PWS individuals has been described as similar (Hoybye et al. 2007) or decreased (Choe et al. 2005), however no study has described overall GI motility in PWS. Gastric rupture and necrosis is a relatively common and devastating consequence of PWS (Wharton et al. 1997; Stevenson et al. 2004; Stevenson et al. 2007). Therefore, examination of the ability of the GI tract to empty in response to meals with varying nutritional components will be necessary to determine if there are actual rather than perceived deficits in GI

motility in PWS individuals. Determining intestinal transit times in *Ndn*-null mice using charcoal or radioactively-labeled food would identify if a defect in gut motility is present in these mice. Studies of the contractility of muscle within the gut of *Ndn*-null mice would also indicate whether necdin is the gene responsible for the suspected reduced gastrointestinal motility in PWS patients.

A number of imaging studies in PWS individuals indicate structural defects in the cortex and pituitary gland (Miller et al. 1996; Miller et al. 2007; Miller et al. 2007; Iughetti et al. 2008; Miller et al. 2008). Post-mortem evaluation of different neuronal populations in the hypothalamus of PWS individuals has been performed in multiple small studies (Swaab et al. 1995; Goldstone et al. 2002; Goldstone et al. 2003; Fronczek et al. 2005). Further examination of post-mortem brain samples in PWS individuals with emphasis on the assessment of the size and location of multiple nuclei in the brainstem should be performed. These nuclei should include the nucleus tractus solitaris due to its role in autonomic regulation of homeostasis and the dorsal motor nucleus as it contains the preganglionic parasympathetic visceromotor fibers. Further histological examination of the cerebellum in PWS individuals should also be undertaken as there is reduced cerebellar volume in these individuals, which to date has unknown consequences but may affect balance and higher cognitive functions. Finally, as serotonin plays a key role in behaviour and breathing deficits in mouse models of PWS, examination of the serotonergic cell bodies in the medulla of PWS brains should be performed to determine if their number or location is different or if axonal outgrowth from these neurons is reduced.

The hypothalamus in the *Magel2*-null mouse appears normal in imaging studies, although there are a reduced number of orexin neurons in the lateral hypothalamus (Kozlov et al. 2007; Mercer et al. 2009). Histological and immunohistochemical examination of other neuronal sub-types in various hypothalamic nuclei has not yet been performed and will be necessary to complete in order to determine whether the survival or differentiation of other neurons is affected by the loss of *Magel2*. Specific nuclei requiring further study

include the arcuate nucleus due to its role in GH secretion and satiety; the paraventricular nucleus as loss of neurons would affect thyroid and adrenal function; and the ventromedial nucleus which is responsible for sensing blood glucose levels and regulating the systemic response to hypo- and hyperglycemia. Identifying deficits in hypothalamic connectivity could be performed by placing retrograde tracers in various nuclei on hypothalamic slices of *Magel2*-null mice and comparing projection patterns to those observed in control littermates. Closer examination of hypothalamic nuclei in *Ndn*-null mice should also be undertaken as the likelihood of abnormalities in the hypothalamus is high due to the differences already described throughout the remaining central nervous system. Necdin's interaction with ARNT2, a transcription factor required for the development of multiple hypothalamic nuclei, also strengthens the possibility of identifying hypothalamic deficits in *Ndn*-null mice. Specifically, it would be interesting to examine the expression of *Brn2*, a downstream target of SIM1/ARNT2 in the hypothalamus, as loss of necdin may adversely affect its expression pattern. Finally, the study of *Ndn* and *Magel2* double null mice would aid in determining the effect that the combined loss of these two MAGE genes have on the development of the hypothalamus, and how their loss contributes to various aspects of PWS.

Growth hormone (GH) treatment in PWS individuals increases final adult height and resolves body composition deficits (Mogul et al. 2008; Carrel et al. 2010). Although the *Magel2*-null mice have increased adiposity and reduced lean mass, their body length is not different than control littermates. Nonetheless, it would be interesting to treat *Magel2*-null mice with GH to determine if their body composition deficits also resolve. To further elucidate the reduced GH response to ghrelin stimulation, it will be necessary to measure GH secretagogue receptor (Ghsr) RNA or protein levels in the hypothalamus and pituitary. A decrease in the level of ghrelin's receptor may explain the reduced response observed versus a complete lack of response as is observed in *Ghsr*-null mice. A reduced GH response may also be caused by altered signaling downstream of GHSR in ghrelin-responsive cells or altered transmission along axons due to the loss of

Magel2. Examining the response of *Magel2*-null Ghsr-expressing neurons to ghrelin using electrophysiological measurements should also be performed to determine which of the above mentioned deficits is more likely.

The identification of an altered counter-regulatory response to hypoglycemia in *Magel2*-null mice indicates the need for examination of this feature in PWS individuals. This could be accomplished by measuring blood glucose levels in PWS individuals and lean and obese controls, throughout the course of the day in addition to recording activities and food intake. It would be interesting to determine if hypoglycemia is more common in PWS individuals overall. If possible, it would also be interesting to start a multi-center trial to examine blood glucose levels in PWS individuals prior to surgeries in which overnight fasting is necessary. This would determine if they are capable of appropriate regulation of blood glucose levels during potential episodes of hypoglycemia. Further evaluation of the counter-regulatory response should also be pursued in *Magel2*-null mice. Examining levels of serum glucagon 30 minutes post-insulin injection would indicate if this aspect of the response is intact since we have determined that the corticosterone response is abnormal in *Magel2*-null female mice but normal in *Magel2*-null male mice. Examining the electrophysiological response of glucose-sensing neurons in the ventromedial nucleus of *Magel2*-null hypothalamic slices to different concentrations of glucose solutions would identify if loss of Magel2 affects action potentials in these neurons.

Although little is known about Magel2's cellular function, by comparing the role of closely related MAGE proteins, including necdin, MAGED1, and MAGEG1, it might be possible to infer a role for Magel2 in neuronal survival, differentiation, or axonal outgrowth leading to altered connections or loss of hypothalamic neurons. Interestingly, *Maged1*- and *Magel2*-null mice both exhibit deficits in circadian rhythm regulation (Kozlov et al. 2007; Wang et al. 2010). Further studies on the role of Magel2 in regulation of circadian rhythm could include examining expression levels of circadian genes including, *Bmal1*, *Clock*,

Per2, and the cryptochrome genes *Cry1* and *Cry2*, in the suprachiasmatic nucleus of the hypothalamus in *Magel2*-null mice at various time points during both the light and dark periods. As circadian changes in gene expression patterns are also observed in the liver, examination of expression of the aforementioned circadian genes in the liver of *Magel2*-null should also be performed, as differences in *Bmal1* levels were identified in both *Maged1*-null liver and suprachiasmatic nucleus (Wang et al. 2010). To determine if there is some functional redundancy between Maged1 and Magel2 in mice, transgenic overexpression of *Magel2* could be induced in *Maged1*-null mice with subsequent examination of the circadian rhythm in adult mice. This would establish whether Magel2 and Maged1 have overlapping functions in the development of the suprachiasmatic nucleus and subsequent regulation of circadian rhythm.

The results presented in this thesis further support a role for necdin and Magel2 in the PWS phenotype. *NDN* and *MAGEL2* are both expressed throughout development, consequently loss of expression of these genes may cause deficits that cannot be reversed. However, with an understanding of the pathways in which necdin and Magel2 are involved, we may be able to identify treatments that modify any differences and return the altered pathways to a normal functioning state. Although growth hormone treatment improves overall health in PWS individuals, new therapeutic goals could include restoring hypothalamic-pituitary axes, eliminating excessive hyperphagia, and restoring aspects of autonomic dysfunction.

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