DISSERTATION

ROLE OF GAMMA-AMINOBUTYRIC ACID (GABA) IN HYPOTHALAMIC NUCLEAR DEVELOPMENT

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY KRISTY MCCLELLAN ENTITLED "ROLE OF GAMMA-AMINOBUTYRIC ACID (GABA) IN HYPOTHALAMIC NUCLEAR DEVELOPMENT" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

ROLE OF GAMMA-AMINOBUTYRIC ACID (GABA) IN HYPOTHALAMIC NUCLEAR DEVELOPMENT

The hypothalamus is involved in energy balance, appetite regulation, stress and anxiety, reproduction including sex behavior, and aggression. It is divided into cell groups based on cell staining, functional similarities and projections. This dissertation looks at the development of three of these cell groups, the ventromedial nucleus (VMN), the paraventricular nucleus (PVN), and the arcuate nucleus (ARC). The VMN and PVN have a unique pattern of GABA expression in elements surrounding the nuclei, with a void of GABA within the region of the developing nuclei. The ARC, on the other hand, has dense GABAergic immunoreactive elements throughout the nucleus. Because of the expression pattern of GABA immunoreactivity surrounding the VMN and PVN, it is likely that GABA is acting as a boundary cue to influence migrating neurons.

Chapter 2 reviews the development of the VMN, including a discussion of the heterogeneity of the nucleus, a description of what cues are involved in cell migration, and descriptive information on the directions of cell movement during development.

Chapter 3 examines the role of GABA on cell migration within the VMN and ARC. A live slice culture system allowed visual tracking of cell movement in the VMN and ARC. There was a difference between the average movement speeds of cells in the VMN vs. cells in the ARC, and upon addition of GABA receptor antagonists to the slices, VMN cells increased in speed while ARC cells did not change. In mice lacking functional $GABA_B$ receptors there was a change in cell positions of neurons containing immunoreactive estrogen receptor (ER)α, which may be due to changes in cell movements and migration. There was no change in amount of cell positions of immunoreactive ERα cells in the ARC.

Chapter 4 looks at the role of GABA and brain derived neurotrophic factor (BDNF) in PVN development. There was a sex-selective effect in $GABA_BR1$ subunit knockout mice of positions of cells containing immunoreactive ERα, indicating that females may be particularly more susceptible to changes in GABA signaling as it may be influencing the final positions of cells. There was also a decrease in BDNF expression in $GABA_BR1$ subunit knockout mice, suggesting that GABA may play a role in cell differentiation.

In conclusion, several lines of evidence indicate roles for GABA in the development of the hypothalamus, in particular, within the VMN and PVN.

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Chapter 1

Introduction

The hypothalamus is located at the base of the diencephalon and is anatomically organized into nuclear groups.

The hypothalamus is a part of the diencephalon, a region of the forebrain involved in the autonomic nervous system, physiological homeostasis, motivated and emotional behaviors, and various other secondary sensory functions. The hypothalamus is involved in energy balance, appetite regulation, stress and anxiety, reproduction including sex behavior, and aggression. In comparison to other regions of the brain, the hypothalamus occupies a relatively small space at the base of the brain bisected by the third ventricle. Studying the development and functions of the hypothalamus is extremely important as there are a number of potential disorders stemming from altered function within various regions of the hypothalamus (Caqueret et al., 2005; Michaud, 2001b). While anatomists have moved away from the notion that individual functions can be isolated to a particular region of the brain, specific behaviors and functions can be impaired or eliminated through neurotoxic lesions destroying a particular region and its connections (Meister et al., 1989). In the hypothalamus, for example, lesions of the VMN cause a loss of sexual behavior (Emery and Moss, 1984). The anatomical organization of the hypothalamus is visible through Nissl stains as individual groups (or nuclei) of cells surrounded by cell-poor zones. These nuclear groups have also been described based on their cellular chemistry, connections, and regulation of specific functions (Canteras et al., 1994; Segal et al., 2005). Each individual nuclear group has specific behaviors and functions associated with it (Canteras et al., 1994; Elmquist, 2001;

Kenney et al., 2003). There are many nuclei described in the hypothalamus but the emphasis of this dissertation is on three: the ventromedial nucleus (VMN), the arcuate nucleus (ARC), and the paraventricular nucleus (PVN).

The diversity of cell phenotypes within the VMN, PVN, and ARC contributes to the regulation of functions and behaviors associated with the hypothalamus.

Nuclear groups can be defined by the molecular homogeneity and heterogeneity of their cellular elements. The VMN is characterized by the expression of the transcription factor steroidogenic factor-1 (Parker et al., 2002), the PVN contains numerous vasopressin and oxytocin synthesizing cells (Armstrong et al., 1980), and the ARC is known for orexigenic and anorexigenic peptides controlling food regulation (Bouret and Simerly, 2004). Conventionally, the ARC has become the primary site for studies on weight regulation, however, disruptions of the VMH can also lead to obesity in mice (King, 2006; Majdic et al., 2002). One focus of chapters 2 and 4 (McClellan et al., 2006, 2008) is to characterize these nuclei in terms of their developmental cytoarchitecture, which includes the expression of certain markers, and the positions of these cells in the embryonic hypothalamus.

The VMN can be subdivided into three components, the dorsomedial, central, and ventrolateral populations of cells (Canteras et al., 1994). The ventrolateral group of cells is involved in the regulation of lordosis behaviors associated with female reproduction in the rodents (Daniels and Flanagan-Cato, 2000; Flanagan-Cato et al., 2001; Manogue et al., 1980). A large majority of these cells have steroid hormone receptors including, estrogen receptor (ER)α, ERβ, androgen receptor, and progesterone receptor (Shughrue et al., 1997; Simerly et al., 1990). The dorsomedial and central region of the VMN contains other markers including SF-1, brain-derived neurotrophic factor, and leptin

receptors (Tran et al., 2006; Tran et al., 2003). The role of SF-1 in the adult VMN has been examined using brain-specific knockouts of SF-1. When SF-1 was selectively disrupted in brain (and thus the VMN), mice exhibited an increase in anxiety-related behaviors (Zhao et al., 2008), which may indicate a role for the VMN in regulating stress and anxiety-like behaviors. Selective disruption of BDNF in the VMN and part of the dorsal medial hypothalamic region caused hyperphagia and a significant gain in body weight in mice. These mice had normal locomotor activity and caloric restriction was able to restore normal body weight (Unger et al., 2007). Another study in rats demonstrated that microinjection of BDNF into the VMN caused a decrease in food intake and body weight gain (Wang et al., 2007). The VMN contains a number of other cell types, many of which are selective to the VMN in comparison to other nuclei within the hypothalamus (Kurrasch et al., 2007; Segal et al., 2005).

The ARC is largely known for having a number of cells that contain leptin receptors, which respond to leptin release from fat cells to regulate appetite. Leptin works to enhance neuropeptide Y (NPY) and agouti-related peptide (AGRP) release from ARC neurons while blocking propriomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) release to increase appetite and decrease energy expenditure (McMinn et al., 2000). NPY, AGRP, POMC, and CART are four peptides synthesized within ARC neurons that are critical in the control of appetite. In humans, most obese patients display an increased level of circulating leptin, which indicates that obesity may be partially contributed to by a state of leptin resistance (Considine and Caro, 1996; Considine et al., 1996). The ARC also contains a dense population of ERα-immunopositive cells, which have been linked to regulating sex behavior (Dewing et al., 2007), along with the VMN, and feeding behavior (Chakraborty et al., 2008), through its connections to the PVN. The ARC also contains a dense population of GABAergic cells

and fibers. It has been suggested that the GABAergic inhibition of neurons of the ARC is very important in regulating the release of orexigenic and anorexigenic peptides (Meister, 2007).

The PVN is one of the more complex nuclei within the hypothalamus in regard to its neurochemistry, connections made, and its diversity based on cell size and type. The rat PVN has been described as having a magnocellular (larger cell bodies within the lateral PVN) and a parvocellular (smaller cell bodies within the medial PVN) division (Armstrong et al., 1980). These divisions are not well defined in mice, although many of the cell types and projections are similar to those seen in rats. Neurosecretory cells of the parvocellular region are comprised of chemically diverse neurons and include cells that are immunoreactive for corticotropin releasing hormone, glucocorticoid receptor, and thyrotropin-releasing hormone among others (Swanson and Sawchenko, 1983). Neurons of the magnocellular PVN largely consist of cells characterized for their content of immunoreactive oxytocin or arginine vasopressin (Armstrong et al., 1980). Other proteins synthesized by neurons in the PVN that are of relevance include BDNF, neuronal nitric oxide synthase, and calbindin. BDNF has been shown to increase energy expenditure and reduce energy intake when perfused directly into the PVN of rats (Wang et al., 2007). In the adult rat PVN, BDNF effectively causes remodeling of synaptic contacts with inhibitory inputs by reducing the number of $GABA_A$ receptor subunits on the membrane surface (Hewitt and Bains, 2006). BDNF also may play a role in the stress response of the hypothalamic pituitary adrenal (HPA) axis within the PVN. Immobilization stress in rats induces BDNF expression in the PVN that precedes increases in CRH and AVP release. BDNF may be a stress-responsive factor influencing the induction of CRH and AVP in the stress response (Givalois et al., 2004).

The VMN, ARC, and PVN are interconnected groups within the hypothalamus. Although these nuclei are often associated with individual behaviors, the connections between the three groups illustrate the complexity of how behaviors are regulated. The VMN, ARC, and PVN are key players in the regulation of neuroendocrine function through connections made within the hypothalamus and throughout the nervous system, particularly downstream to the autonomic nervous system. Intrahypothalamic connections exist between the VMN, ARC, and PVN as well as between other hypothalamic regions.

The major afferent projections to the VMN are from the amygdala, a part of the limbic system, and ventral subiculum (Canteras and Swanson, 1992), which is involved in the regulation of the HPA axis (Herman et al., 1998). These afferents innervate the ventrolateral part of the VMN. The amygdala projects to the dorsomedial region. The VMN receives intrahypothalamic inputs from the medial preoptic area, anterior hypothalamus, posterior hypothalamic region, and the suprachiasmatic nucleus (Fahrbach et al., 1989; Saper et al., 1976). The major afferents from the brain stem come from the parabrachial nucleus. Efferents from the VMN are largely intrahypothalamic; however there is a large projection to the dorsal midbrain from neurons in the ventrolateral VMN (Morrell and Pfaff, 1982).

The PVN is subdivided into magnocellular and parvocellular cells. Neurosecretory cells of the parvocellular PVN have inputs to the capillary loops of the median eminence (Armstrong and Hatton, 1980; Kawano et al., 1991). Parvocellular neurons can also project to extrahypothalamic regions. Axons project laterally towards the fornix and arc before turning towards the ME (Swanson et al., 1986)(Swanson et al., 1986, Rho and Swanson, 1989).

A group of PVN neurons projects caudally towards the brain stem and spinal cord (Hoysoya and Mutsushita, 1979, Swanson and Kuypers, 1980). These projections come from neurons in the dorsomedial cap, ventral PVN, and posterior PVN. The dorsomedial cap projects to the intermediolateral cell column and the ventral PVN, and the posterior PVN sends projections to the spinal cord and other brainstem nuclei (dorsal motor nucleus of the vagus, periaqueductal gray, dorsal raphe nuclei, locus coeruleus, parabrachial nucleus, reticular nucleus) (Saper et al., 1976, Luiten et al., 1985). Other pathways include projections to the thalamic PVN, medial and central amygdala (Larsen et al., 1991), AVPV, anterior hypothalamus, VMN, ARC, and PF regions (Luiten et al., 1985, ter Horst and Luiten, 1987, Larsen et al., 1991).

Intrahypothalamic projections to the PVN and VMN have been traced using phaseolus vulgaris Leuco-Agglutinin (TerHorst et al., 1986). This group has shown that projections from the VMN produce labeled projections in the areas that ventrally and dorsally surround the PVN, however, these fibers do not synapse on cell bodies within the PVN. The lateral hypothalamic area has dense inputs to the magnocellular division of the PVN with few inputs to the parvocellular region. Intranuclear connections within the PVN arise from parvocellular cells synapsing on parvo and magnocellular cells; however, magnocellular neurons do not appear to project to parvocellular cells. Based on anterograde labeling using phaseolus vulgaris leuco-agllutinin, intranuclear connections within the PVN appear to be much less abundant than within the VMN (TerHorst et al., 1986).

In the VMN, there are few projections from the lateral hypothalamic area. A dense fiber innervation comes from the dorsomedial hypothalamus. Input from the parvocellular PVN synapses in the medial aspect of the VMN at all longitudinal levels. The

magnocellular PVN does not appear to project to the VMN. The largest density of intrahypothalamic projections to the VMN comes from the anterior hypothalamus (TerHorst et al., 1986). DiI labeling of fibers from the preoptic area shows a dense collection of fibers surrounding the VMN (Budefeld, Tobet, and Majdic; personal communication) where there is an extensive field of VMN dendrites that might collect information (Crandall et al., 1989; Flanagan-Cato et al., 2001).

The arcuate nucleus is a particularly important site for the central regulation of food intake, body weight, and energy expenditure. Neuronal loss in the ARC has been linked to obesity syndromes characterized by increased food intake (Meister et al., 1989, Debons et al., 1982, Bergen et al., 1998). Projections from the ARC innervate other hypothalamic nuclei involved in the control of feeding which include the PVN, dorsomedial and lateral hypothalamic area (Elias et al., 1998, 1999; Elmquist et al., 1998). ARC projections also innervate regions of the midbrain, some of which are involved in nociception (Sim et al., 1991).

Forming a functional nuclear group requires a series of steps leading from final cell divisions to the establishment of axonal connections between cells.

Those who study development might generally categorize it into five main stages; cell proliferation, cell migration, cell differentiation, developmental or programmed cell death, and axon guidance and the establishment of synaptic connections (Sanes et al., 2000). Cell proliferation/ neurogenesis occurs along the subventricular zone (specifically the third ventricle in hypothalamus), where neuronal precursor cells are dividing and beginning to differentiate (Dehay and Kennedy, 2007). Cell migration takes place when a cell goes through its final division and moves away from the ventricle towards its final location in the brain (Marin and Rubenstein, 2003). Cell differentiation or cell fate

determination can happen at various time points throughout development. Cell fate determination can take place early in proliferating cells or throughout migration. Some cell types (e.g. ERα) seem to differentiate (or start the synthesis of estrogen receptors) only after reaching the location where they are found in the adult (e.g. the vlVMN; chapter 3). Cell death occurs throughout the brain during development, and plays a role in the organization of structures (Buss et al., 2006). Axon guidance and the establishment of synaptic connections appear to be, for the most part, the final stage of development. It occurs when cells send axonal projections to various other brain nuclei. These can be intrahypothalamic or extrahypothalamic connections as described above for the VMN, ARC, and PVN. The next three chapters focus on the early stages of the development of the VMN, ARC, and PVN; when cells are proliferating and migrating from the third ventricle. The importance of studying the development of these nuclei is extremely relevant to understanding more about normal development so that we are able to recognize when processes in development occur abnormally, to determine which factors are important in these developmental processes, and to determine how these factors may be altered by endogenous, environmental or pharmacological substances.

The neurochemistry of developing hypothalamic nuclear groups may give insights into the factors involved in the development of these regions.

Prior to the establishment of projections that synapse and functionally connect these hypothalamic nuclear groups, numerous transcription factors, peptides, and neurosecretory molecules are being synthesized within the hypothalamus. It's been suggested that these factors have functions in development differing from their roles in the adult brain. It has been shown that the expression of molecules in development may play a role, different from their known roles in adulthood. For example, steroid hormones (McCarthy, 2008), leptin (Bouret and Simerly, 2004) and BDNF are three factors whose

role can change between early development and adulthood. It has also been shown that patterns of expression (especially patterns unique to individual nuclei) correspond to important developmental roles for some transcription factors. SF-1, a factor that is only expressed in cells of the VMN within the brain, is critical in normal VMN development. SF-1 knockout mice have altered distributions of cells in the region of the VMN and when raised to adulthood these mice become obese, a further indicator of the role of the VMN in body weight regulation (Majdic et al., 2002). The transcription factor Sim-1 may play similar role(s) in the PVN. Although it is less clear, Sim-1 was originally implicated in the process of cell differentiation for certain neuronal phenotypes (Michaud et al., 1998b), however, it also may be involved in altering the cellular positions within the PVN (Xu and Fan, 2007).

The pattern of GABA expression in the developing hypothalamus suggests that it may play a role in providing boundary information for the development of the VMN and PVN. Previous work in our lab has shown that the distribution of cells and fibers that synthesize and contain GABA surrounds the developing VMN (Tobet et al., 1999), and more recently we have shown this pattern in and around the developing PVN. All other areas of the embryonic hypothalamus, including the ARC, contain numerous cells or fibers that can synthesize or contain GABA. This unique pattern of GABA immunoreactivity surrounding these nuclear groups implicates GABA as having a role in the developing hypothalamus, possibly through providing boundary information for the areas that will become the ventromedial and paraventricular nuclei.

GABA has been found to be an important factor in development with many roles including its ability to influence migrating neurons. GABA's influence on migration is best understood in the cerebral cortex. GABA induces migration of embryonic cortical

neurons in vitro and activation of GABA receptors can stimulate migration to cortical regions of the developing brain (Behar et al., 1996; Behar et al., 2000; Behar et al., 1998). GABA's influence on migration can work through both its A and B receptors. Specific antagonists have been used to show that blocking $GABA_B$ receptors appears to increase the number of tangentially migrating neurons and decreases the length of dendrites (Lopez-Bendito et al., 2003). $GABA_B$ receptors are highly expressed on cells in several layers of the cortex, but much less is known about migration to other regions of the brain that express $GABA_B$ receptors.

GABA's main role in adulthood is as an inhibitory neurotransmitter, however, in development GABA can be excitatory, causing depolarization. In cultured embryonic (E15-E18) hypothalamic neurons, GABA immunoreactivity is found throughout neurons, processes, and at the growth cone indicating it's presence prior to synapse formation (van den Pol, 1997). It has also been shown that GABA is released from growth cones through vesicular exocytosis and stimulating action potentials can evoke release of GABA from the growth cone (Gao and van den Pol, 2000). In developing brain slices and cultures of hypothalamic neurons, most action potentials are due to GABA release from developing axons. In later development, depolarizing action potentials arise mainly from glutamate release while GABA is the main transmitter involved in hyperpolarization of a cell (Gao and van den Pol, 2001). GABA acts through its receptors $GABA_A$, $GABA_B$, and $GABA_C$. Both $GABA_A$ and $GABA_C$ are ion channel receptors regulating the flux of chloride within a cell. These ion channels work quickly to modulate the effects of the neurotransmitter GABA. $GABA_B$ receptors are metabotropic, working through second messenger systems to regulate neuronal activity. $GABA_B$ receptors are classic G-protein coupled receptors consisting of a seven transmembrane domain-spanning region. This specific G-protein mechanism involves coupling to G_i and G_o proteins, which in turn

inhibit the effector enzyme adenylate cyclase to produce downstream effects. Presynaptic $GABA_B$ receptors couple to Ca^{++} channels inhibiting Ca^{++} flux while postsynaptic $GABA_B$ receptors couple to K^+ channels resulting in hyperpolarization. $GABA_B$ receptors are heterodimers composed of two subunits, R1 and R2. $GABA_BR1$ is retained in the plasma membrane (Couve et al., 2000) until heterodimerization with $GABA_BR2$, trafficking the protein to the cell surface (Jones et al., 1998; Kaupmann et al., 1997; Kuner et al., 1999). There is some controversy arguing that heterodimerization of the subunits is unnecessary to form a functional receptor, but studies show that only $GABA_BR1$ can bind to ligands (Kniazeff et al., 2002) making it necessary to have both subunits to have a functional receptor.

$GABA_A$ and $GABA_B$ receptors are expressed within the cells of the VMN as early as E13 (Dellovade et al., 2001; Davis et al., 2002) but GABA itself is localized within fibers and cells that surround the VMN (Tobet et al., 1999; see Fig 2). SF-1 is important in setting up the VMN cytoarchitecture during the early stages of development (Dellovade et al., 2000, Davis et al., 2004; reviewed McClellan et al., 2006; chapter 2), but it is likely that GABA is important in the formation of the VMN in the middle and later stages of development. $GABA_A$ (Laposky et al., 2001) and $GABA_B$ (Prosser et al., 2001) receptor knockout mice had been created and were acquired to study the effects of GABA signaling on VMN formation. $GABA_A$ receptor subunit $\beta 3$ knockout mice show a change in the distribution of cells containing immunoreactive $ER\alpha$ (Dellovade et al., 2001) similar to the results obtained with $GABA_B$ receptor knockout mice (McClellan et al., 2008; chapter 3). This data suggests that GABA, through its receptors acts as a tropic factor, setting up boundaries for the cells that occupy the ventrolateral portion of the VMN. The $GABA_B$ receptor agonist baclofen decreased the rate at which cells of the VMN migrated but did not change the likelihood of any particular cell to move (Davis et al., 2002). The

next 3 chapters discuss the utilization of a $GABA_B$ receptor knockout mouse to further delineate GABA's effect on migration in the VMH. The $GABA_BR1$ knockout mice die at around 25 days of age from epileptic seizures and exhibit a lower body weight as compared to littermate controls. Another group generating a knockout of the same gene found it to have a heightened sensitivity to pain, memory impairment, and a decrease in anxiety (Schuler et al., 2001).

Chapter 2

Development of the Ventromedial Nucleus of the Hypothalamus

Abstract

The ventromedial nucleus of the hypothalamus (VMH) is important in the regulation of female sexual behavior, feeding, energy balance, and cardiovascular function. It is a highly conserved nucleus across species and a good model for studying neuronal organization into nuclei. Expression of various transcription factors, receptors, and neurotransmitters are important for the development of this nucleus and for mapping the position of identified cells within the nucleus. The VMH is subdivided into regions, all of which may project to specific locations to carry out various functions. For example, the ventrolateral quadrant contains a subset of neurons that highly express estrogen receptors. These neurons specifically, are involved in the lordosis response pathway through projections to other estrogen receptor containing regions. In development, neurons that form the VMH generate from the proliferative zone surrounding the third ventricle. Neurons then migrate along radial glial fibers to final positions within the nucleus. Migration and positioning of neurons is an important step in setting up connections to and from the VMH and hence in its function. As compared to other developing brain regions, cell death may play a minor role in sculpting the VMH. We review the processes involved in forming a functional nuclear group and some of the factors known to be involved particularly focusing on the positioning of identified neurons within the VMH.

1. Introduction

There are two major organizational schemes for neurons in the vertebrate central nervous system: layers (e.g., cerebral cortex) and nuclei (e.g., diencephalon). There is a growing understanding of cortical development, and a number of the molecules that play roles in this process have been identified (Grove and Fukuchi-Shimogori, 2003; Lambert de Rouvroit and Goffinet, 2001; Marin and Rubenstein, 2001; Marin and Rubenstein, 2003; Nadarajah and Parnavelas, 2002), however; significantly less is known about the development of nuclear groups (Letinic and Rakic, 2001; Michaud, 2001a; Simerly, 2002; Tobet, 2002). The proposed "protomap" for cortical regions involves newborn cells arising from proliferative zones surrounding the lateral ventricles and developing into cortical layers (Rakic, 1988; Rakic, 1995). In the hypothalamus, particular regions along the third ventricle give rise to cells forming specific subdivisions (Altman and Bayer, 1986).

Progress in delineating broad compartments (e.g. prosomeres) within the forebrain has emerged from experiments describing the distribution of transcriptional regulating genes (Rubenstein and Beachy, 1998) and other factors that either mediate (Ganzler and Redies, 1995; Gotz et al., 1996), or induce (Ericson et al., 1995) ventral forebrain organization. Prosomeres are transverse domains defined by certain morphological and molecular similarities. Such compartments may indicate groups with shared cell histories and common influences (Puelles and Rubenstein, 1993). Apparent boundaries for compartments of these cell groups may be delineated by the presence of cell dense zones surrounded by cell poor zones (Christ, 1969), and comprised of fiber bundles (Silver et al., 1993) or selective molecular environments (Steindler and Cooper, 1987). Numerous studies suggest that these regions of cell density also modulate selected functions such as circadian rhythms by the suprachiasmatic nucleus (Saper et al., 2005),

or stress responses by the paraventricular nucleus (Kenney et al., 2003). Understanding how cells position themselves into a nucleus that regulates particular physiological functions or behaviors has been hindered by the complexity of the processes involved (Tobet, 2002).

The development of a hypothalamic nuclear group begins with neurogenesis in the proliferative zone of the third ventricle. This requires divisions that yield progenitor cells, followed by divisions that yield terminally mitotic cells that exit the proliferative zone surrounding the third ventricle to take up residence in the hypothalamus. While evidence suggests that cell fate information may be imparted during neurogenesis in the cortex (McConnell, 1995), this is less established for hypothalamic structures. In one study, the development of estrogen receptor-containing neurons was found to proceed autonomously following the transplantation of fetal hypothalamic tissue into different environments (Paden et al., 1985). Although an anatomical technique was used to evaluate estrogen receptors (autoradiography), the tissue was evaluated more for the presence of estrogen binding than for the formation of particular nuclear groups. Nonetheless, the data are consistent with an early determination of cell fate. Following a final mitotic event, a cell must migrate (or not) to a destination among potential hypothalamic destinations to join particular cell groups. Finally, as a fully differentiated neuron, axons are projected to other sites in the CNS and synaptic input is received from other sites, thereby facilitating specific functions or behaviors. This review follows the development of the ventromedial nucleus of the hypothalamus (VMH, sometimes referred to as VMN) as the nucleus emerges from an undifferentiated cell mass.

The VMH is a medial hypothalamic cell group that sits close to the base of the diencephalon, adjacent to the third ventricle above the median eminence and pituitary

complex. It is a bilateral cell group that has an elliptical shape, stretching more laterally in rodents as it extends rostral to caudal (Fig. 1A). The VMH is first clearly defined as a nuclear group between E15 and E17 in the mouse (Tobet et al., 1999) (Fig. 1B). Its cytoarchitecture is strongly defined by the surrounding cell-poor, fiber-rich, zone. The cell-poor zone is rich in dendritic processes (Crandall et al., 1989; Millhouse, 1973), providing an extensive receptive surface for fibers of the stria terminalis (Heimer and Nauta, 1969). Within the VMH, subnuclear groups have been delineated based on fiber projections and cell types, including dorsomedial, central, ventrolateral, and anterior zones (Canteras et al., 1994; Saper et al., 1976; Van Houten and Brawer, 1978). A basal subnucleus in the ventrolateral region, designated the tuberal nucleus, also can be delineated based on neuronal birthdates and cell phenotype (Altman and Bayer, 1986; Canteras et al., 1994; Whorf and Tobet, 1992).

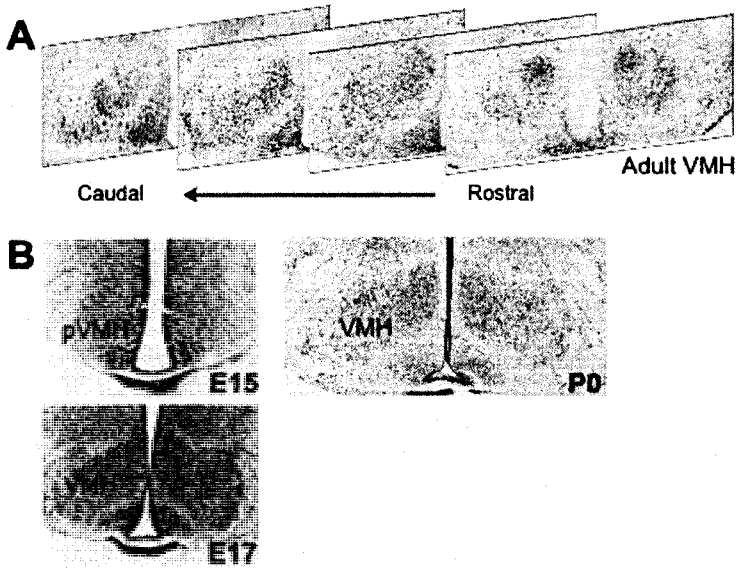

Fig. 1. Images of Nissl stained sections of the adult mouse VMH (A) through 4 coronal planes moving rostral to caudal. Rostrally the VMH appears as a circular nucleus adjacent to the ventricle and stretches ventrolaterally in a more oblong shape moving caudally through the nucleus. The developing VMH has a similar shape (B). It is not clearly discernible at embryonic day 15, but begins developing distinct boundaries by E16 – E17. By P0 there is a distinct nucleus resembling that of the adult.

The VMH has been implicated in a broad array of homeostatic and behavioral functions, including affective, ingestive and sexual behaviors (Canteras et al., 1994). Conventionally, the arcuate nucleus has become the primary site for studies of weight regulation and feeding behavior; however, disruptions of the VMH can also lead to obesity in adulthood (Majdic et al., 2002). As we learn more of the molecular nature of cells within the VMH, and of signaling molecules inside and outside of the nucleus, we are gaining a greater appreciation for specific cell types and molecules in the VMH that may play role(s) in regulating feeding behavior or energy balance (Ohata et al., 2000). For a more thorough review of hypothalamic pathways involved in feeding behavior see reference (Elmquist, 2001). The VMH is also important for the regulation of female sexual behavior. In particular, the lordosis response is dependent upon neurons in the ventrolateral VMH, specifically those that express estrogen receptors. Electrolytic lesions that involve ventrolateral VMH neurons containing steroid hormone receptors (e.g., estrogen and progesterone receptors) inhibit lordosis behavior in both rats (Emery and Moss, 1984), and cats (Leedy and Hart, 1985). The important complementary result – activation of behavior following steroid hormone implantation directly to the VMH – has also been shown (Rubin and Barfield, 1983). Cardiovascular function can also be regulated by the VMH; stimulation of neurons within the VMH can cause a change in blood pressure and/or heart rate (Hirasawa et al., 1996). The VMH also has a role in the pain pathway. Electrical stimulation of the VMH induces analgesia in rodents (Culhane and Carstens, 1988; Duysens et al., 1989; Rhodes and Liebeskind, 1978) and disruptions of the VMH cause hyperalgesia in rats (Mukherjee et al., 2002). A proposed mechanism for this effect includes the binding of prostaglandin E2 to its EP1 receptor, stimulating an analgesic pathway (Hosoi et al., 1999). The remainder of this review focuses on the structural and neurochemical development of the VMH rather than in adulthood.

The VMH has been thought of as a collection of heterogenous cell types, some of which have been identified. The identification of populations of neurons has been a focus for several studies. One recent study (Segal et al., 2005) used laser-capture microdissection to isolate a set of genes that is enriched in the VMH relative to surrounding areas. Ongoing studies will help identify various genes expressed in different subsections of the VMH. The discovery of the gene steroidogenic factor-1 (SF-1) (Hatano et al., 1994; Honda et al., 1993; Ikeda et al., 1994) as a gene expressed selectively within the VMH, has ushered in a new way of thinking about the structure, function and development of this important hypothalamic cell group. This review will discuss the developmental processes leading to a functional VMH and will categorize molecular factors related to its development through neurogenesis, migration, differentiation, and connectivity.

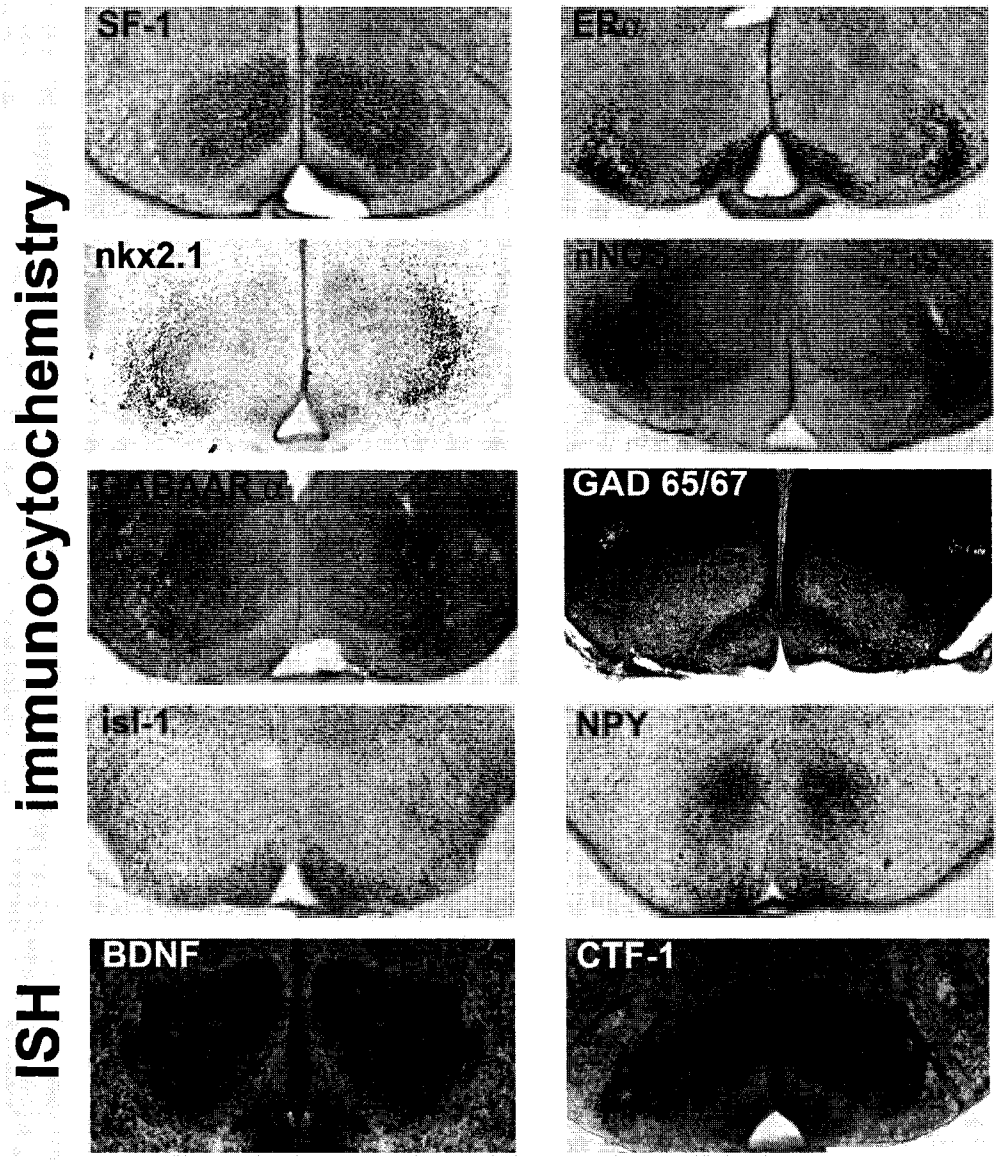

Fig. 2. Images of gene expression patterns of various proteins and mRNA in coronal sections containing the VMH at P0 in mice. Sections containing immunoreactive SF-1, ERα, nkx2.1(TTF-1), nNOS, $GABA_A$ R α1 subunit, GAD 65/67, islet-1, and NPY were run for immunocytochemistry using previously published methods (Davis et al., 2004b; Dellovade et al., 2000; Henderson et al., 1999; Tobet et al., 1999). BDNF and Coup TF1 (CTF-1) riboprobes were used for in situ hybridization as adapted from the Boehringer Manheim digoxygenin system (Boehringer Mannheim, 1995; Dellovade et al., 2000). Antiserum directed against SF-1 was generously provided by Dr. Ken Morohashi. The CTF-1 riboprobe was generously provided by Dr. M.-J. Tsai. Antiserum directed against the $GABA_A$ receptor α1 subunit was obtained from Phosphosolutions Inc. (Aurora, CO).

2. Cell Identities

Cell identity is an important characteristic of brain organization. Phenotypically identified cells in the hypothalamus during development can be found in patterns or domains that can be seen across vertebrate species (Puelles and Rubenstein, 1993; Puelles and Rubenstein, 2003). The VMH contains neurons and fibers that can be grouped by specific cell types into subnuclear regions (e.g., dorsomedial, central and ventrolateral). Factors expressed in cells of the VMH may be potential candidates for developmental signals or cues that can aid in understanding how an undifferentiated neuron becomes a differentiated component of a hypothalamic nucleus. The VMH can be subdivided based on the expression of various proteins (Fig. 3). For example, gonadal steroid hormone receptors help define zones within the VMH. Thus, a population of cells containing estrogen receptors (ER) α and β characterize the ventrolateral quadrant of the VMH, while androgen receptors characterize the nucleus in general and a basal subnucleus in particular (Shughrue et al., 1997; Simerly, 2002). The expression of the transcription factor SF-1 (Honda et al., 1993; Ikeda et al., 1994; Morohashi et al., 1993; Roselli et al., 1997) delineates cells in the medial and central regions of the VMH in ways that are important for new studies of VMH structure, function, and development. For a schematic depiction of one way to visualize subdivisions of the VMH see Figure 8. Table 1 and Fig. 2 provides a partial list of factors that are expressed in the VMH and gives their expression patterns within the nucleus.

2.1 Steroidogenic Factor 1 –Brain expression restricted to the VMH

SF-1 (officially designated NR5A1) is currently unique among transcription factors expressed in the brain because its expression is restricted to cells in the VMH of embryonic and adult mice and rats. It is encoded by the *FTZ-F1* gene, an orphan nuclear receptor superfamily member that regulates the expression of the pair-rule gene *fushi*

tarazu in Drosophila (Lavorgna et al., 1991). Rodent homologs of this gene identified as adrenal-4-binding protein (Ad4BP) (Honda et al., 1993) in rats, and SF-1 (Lala et al., 1995) in mice, regulate the expression of many genes that play key roles in steroidogenesis and reproduction, including the atypical nuclear receptor Dax-1 (Kawabe et al., 1999). Immunoreactive SF-1 has been found in the mouse diencephalon as early as embryonic day (E)9.5 (Ikeda et al., 2001). In neonatal rat brain, it was suggested that immunoreactive SF-1 was excluded from the ventrolateral portion of the VMH that contains cells rich in immunoreactive estrogen receptors (Shinoda et al., 1995); and analyses in mice confirm this impression (Dellovade et al., 2000). Strikingly, the first studies of a targeted disruption of the Ftz-F1 genetic locus (Ikeda et al., 1995; Luo et al., 1995; Shinoda et al., 1995) showed a severe disruption of the VMH as defined by its cytoarchitecture based on Nissl stains. SF-1 is also expressed in the adrenals and gonads, proving to be important in the development of these structures as well (Hanley et al., 2000; Luo et al., 1995). Generation of a mouse lacking the SF-1 receptor emphasized the importance of this protein for survival. Mice lacking SF-1 were unable to survive after birth due to the lack of adrenal formation (Ikeda et al., 1995; Luo et al., 1995; Shinoda et al., 1995) and analysis of the brains of these knockout animals at birth or younger also shows that SF-1 is important in establishing the cytoarchitecture of the VMH. In SF-1 knockout mice, cells with identified molecular phenotypes are displaced to other positions within and outside of the nucleus (estrogen receptors, nkx2.1, galanin, etc.) (Davis et al., 2004a; Dellovade et al., 2000; Shinoda et al., 1995) and/or the expression of specific genes is significantly suppressed (BDNF) (Majdic et al., 2002; Tran et al., 2003). The global SF-1 knockout mouse does not survive into adulthood due to missing adrenal glands. Neonatal glucocorticoid therapy and adrenal transplants in these knockout mice, however, allow them to survive into adulthood

(Majdic et al., 2002). These mice become obese in adulthood and ongoing studies are looking at other behavioral or neurochemical differences in these mice.

2.2 Cells of the VMH are characterized by the expression of several transcription factors, receptors, and neuropeptides

In addition to SF-1, other transcription factors are highly expressed within the VMH, albeit with significant expression in other locations as well. One transcription factor nkx2.1 (also known as thyroid transcription factor-1; TTF-1) is known to be critical for hypothalamic differentiation (Kimura et al., 1996a; Sussel et al., 1999) and has been implicated in hypothalamic mechanisms of puberty (Lee et al., 2001). Genetic disruption of nkx2.1 results in a severe disruption of the medial basal hypothalamus such that nuclear groups are no longer recognizable (Kimura et al., 1996a). Islet-1 (isl-1), another transcription factor localized to the VMH, may be important in development based on the circumstantial evidence of its expression pattern. It is found in populations of differentiating neurons, is one of the earliest markers of developing motor neurons (Tsuchida et al., 1994), and is found in cells of the pancreas and endocrine system (Karlsson et al., 1990). Isl-1 expression is a marker for nuclear development in the developing hypothalamus, including the VMH (Davis et al., 2004b). Neuropeptide-Y (NPY) is highly expressed in cells and fibers of the arcuate nucleus that stretch to the paraventricular nuclei, where it acts as a potent orexigenic stimulus. Immunoreactive NPY is also found in cells dorsal to the VMH but is particularly notable in fibers that travel through or terminate in the VMH in rodent (Dellovade et al., 2000) and sheep (Muhlhausler et al., 2004), potentially playing a role in feeding circuitry and other behaviors. Coup transcription factors are orphan nuclear receptors of the steroid/thyroid superfamily. Coup-TF1 expression in the basal telencephalon has been suggested to promote cell migration from the lateral ganglionic eminence to the preoptic area of the

hypothalamus (Tripodi et al., 2004). This influence on migration may be through an integrin-dependent mechanism (Adam et al., 2000). Nonetheless, we (S.A. Tobet, unpublished observations) have been unable to demonstrate reliable differences in cell positions or nuclear grouping in the preoptic area or VMH in brains from Coup-TF1 knockout mice generously provided by Dr. M.J. Tsai (Baylor College of Medicine, Houston, TX).

Steroid hormone receptors are expressed in stereotypical locations within the VMH in many species, including the human (Fernandez-Guasti et al., 2000; Kruijver et al., 2003), mouse (Davis et al., 2004b), rat (Ikeda et al., 2003), ferret (Tobet et al., 1993), guinea pig (Warembourg, 1977), rabbit (Caba et al., 2003), amphibian (Wennstrom et al., 2003) and shrew (Dellovade et al., 1992). As stated above, estrogen and progesterone receptors are localized strongly to the ventrolateral regions of the nucleus, while androgen receptors are expressed throughout the nucleus (Simerly et al., 1990). A number of studies have shown that the neurons within the VMH containing steroid hormone receptors, particularly those of the ventrolateral region, are involved in regulating female sex behavior in adult animals (Calizo and Flanagan-Cato, 2003; Daniels and Flanagan-Cato, 2000; Delville and Blaustein, 1991; Greco et al., 1998; Mani et al., 1994).

New approaches have detected other proteins that may be expressed more exclusively in the VMH than in other regions. Using laser-capture microdissection followed by microarray analysis, a group of factors was recently localized to the VMH; these include cerebellin1, PACAP, slit3, and a novel protein termed LBH2 (named after the limb bud and heart development family of factors) (Segal et al., 2005). This information verifies other approaches that have found PACAP (Anderson et al., 2005; Zhou et al., 2000) and

slit (Erskine et al., 2000) proteins to be expressed in the region of the VMH. Slit as a peptide ligand for robo receptors has been shown to act as a repellent for growth cone extension (Lin et al., 2005; Stein and Tessier-Lavigne, 2001) or cell migration (Nguyen-Ba-Charvet et al., 2004) in different systems during development. Although slit proteins are localized to the VMH, no robo receptors for this ligand have been shown to be in the VMH. Perhaps slit protein expression in the developing VMH precludes entry of particular axons coming from other regions that express the robo receptor. Another approach used a large scale screening process to identify transcription factors expressed in the brain and to characterize their expression (Gray et al., 2004). Using polymerase chain reaction and in situ hybridization, the expression domains of hundreds of transcription factors in the developing mouse brain have now been defined (http://mahoney.chip.org/mahoney/). Using this method, Sox14, a member of the Sry-related Sox transcription factor family, was found to be highly expressed in the VMH (Gray et al., 2004). Sox14 has been implicated in both the dorsoventral cell-type specification of spinal cord neurons (Hargrave et al., 2000) and limb development (Wilmore et al., 2000). Additionally, strong expression of Vax1 was noted in the VMH (Gray et al., 2004). Vax1 is a homeobox-containing gene, identified in the mouse and human, closely related to other gene families (Not and Emx) that are required for the formation of structures in which they are expressed. While forebrain localization was noted previously (Hallonet et al., 1998), a strikingly selective expression in the VMH was not quite as strongly in evidence.

2.3 Cellular identities of the VMH – role in signal transduction

Other cell-specific phenotypes within the VMH are based on the expression of components of particular signal transduction pathways. These may influence development through interactions between specific ligands and their receptors. GABA

receptors are one important family of receptors that are expressed in cells of the VMH. There are three types of GABA receptors; $GABA_A$, $GABA_B$, and $GABA_C$. Both $GABA_A$ and $GABA_C$ are ion channel receptors regulating the flux of chloride across the plasma membrane. $GABA_A$ receptors are each made up of 5 subunits and at least 20 possible subunits have been cloned to date (Cherubini et al., 1991; Laurie et al., 1992; Wisden et al., 1992). These ion channels rapidly mediate the effects of the neurotransmitter GABA. $GABA_B$ receptors are metabotropic, working through second messenger systems to regulate neuronal activity. $GABA_B$ receptors are classic G protein-coupled receptors that couple to G_i and G_o proteins, which in turn inhibit the effector molecule adenylyl cyclase to produce downstream effects (Couve et al., 2000). They are heterodimers comprised of two subunits, $GABA_BR1$ and $GABA_BR2$. $GABA_B$ receptor subunits are located in the dorsomedial and central regions of the VMH, while the $GABA_A$ receptor subunits are located in various subpopulations within the nucleus. Fig. 2 shows the $GABA_A$ receptor subunit $\alpha 1$ and its expression pattern within the VMH. $GABA_A$ and $GABA_B$ receptors are expressed in the nascent VMH at least as early as E13 (Davis et al., 2002; Dellovade et al., 2001) and persist into adulthood. Glutamic acid decarboxylase GAD (forms 65 and 67), the biosynthetic enzymes for GABA, are not expressed in cells of the VMH (Okamura et al., 1990). However, GAD expression is high in cells surrounding the VMH (Fig. 2 and Fig. 3) (McCarthy et al., 1995; Okamura et al., 1990) and fibers containing GAD and GABA surround the VMH prenatally and strongly enter the VMH after birth (Tobet et al., 1999). These expression patterns indicate that GABA may be having an effect in establishing the boundaries of the VMH.

In addition to adenylyl cylase, guanylyl cyclase may also play a role in signal transduction of the VMH. Neuronal nitric oxide synthase (nNOS) catalyzes the production of nitric oxide (NO) and citrulline from arginine. NO is a gas that also serves

as a chemical signaling molecule in the brain, involved in synaptic transmission and plasticity. NO diffuses quickly from its site of production and influences soluble guanylyl cyclase, leading to the formation of cyclic GMP, an important second messenger in signal transduction. Immunocytochemistry for nNOS reveals that it has a unique expression pattern within the VMH. It is expressed in the ventrolateral region and on the lateral edges of the central VMH (Fig. 2). NO has been shown to regulate cell proliferation and differentiation in the development of the vertebrate nervous system (Arnhold et al., 2002; Peunova and Enikolopov, 1995; Peunova et al., 2001) and may be playing this same role in the hypothalamus. As of yet there is no evidence indicating a role for NO in the VMH, however, its expression patterns in development lead us to suggest that it may be involved.

Another receptor highly expressed in VMH neurons is the Cannabinoid Receptor 1 (CB-1). The CB-1 receptor is a G protein-coupled receptor whose ligands are the endogenous cannabinoids, including arachidonic acid metabolites (e.g. anandimide, 2-AG, noladin ether), as well as exogenous plant compounds (e.g. tetrahydrocanabinol). CB-1 is highly expressed in a number of brain regions (Howlett et al., 2004); however, its expression in the hypothalamus is strikingly localized within the boundaries of the VMH. In the human brain, CB-1 receptors are expressed as early as 19 weeks gestation in the same regions where they are expressed in the adult (Mato et al., 2003). In the rat, CB-1 receptors are expressed as early as E14 and are specifically localized to the VMH at E18 (Berrendero et al., 1998b). The presence of these receptors in development suggests a potential role in cell signaling during VMH formation and differentiation.

Table 1 – Cell identities and locations within the VMH.

Cell identities	Region	Reference number
CRF-2	DM, C, VL	[23,118,176]
COUP TF-1	DM, C, VL	[171,183] *
Islet-1	C, VL	[35,36]
Nkx2.1 (TTF-1)	C, VL	[35,125]
Cerebellin 1	DM, C, VL	[152]
Sox 14	DM, C, VL	[58]
SF-1	DM, C	[103]
Estrogen Receptor α	VL	[41,184]
GHSR	DM, C	[61,83]
$GABA_A$ receptors	DM, C, VL	[40]
$GABA_B$ receptors	DM, C, VL	[34]
Androgen Receptor	DM, C, VL	[158]
CB-1	DM, C, VL	[13]
BDNF	DM, C	[162]
Neuropeptide Y	DM, C	[41,177]
Enkephalin	VL	[3,65,142]
Somatostatin	VL	[70]
Substance P	VL	[128]
Oxytocin receptor	VL	[29,81]
cholecystokinin	VL	[2]
PACAP	DM, C, VL	[152,186]

DM, dorsomedial; C, central; VL, ventrolateral

*S.A. Tobet, A.M. Davis, J.R. Masterson, H.J. Walker, E.P. Bless, F.A. Periera, M.J. Tsai; unpublished data presented at Meeting of Society for Neuroscience, 2001.

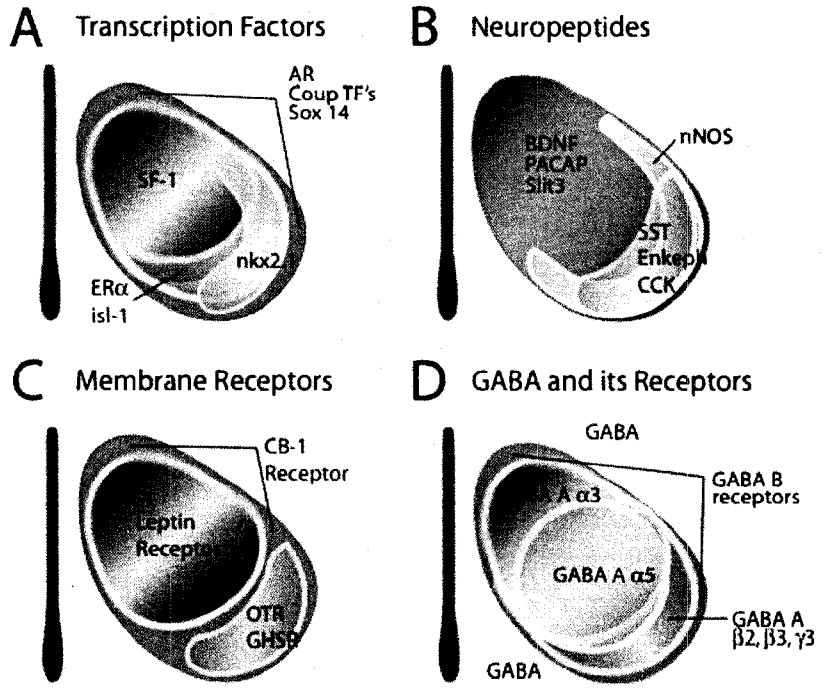

Fig. 3. Schematic diagram showing the expression patterns of various transcription factors (A), neuropeptides (B), membrane receptors (C), and GABA receptor subunits (D) in the VMH. The VMH can be subdivided into regions based on the expression of various factors. This schematic demonstrates the heterogeneity of the nucleus during development and how factors overlap in their expression. In panel A, androgen receptors (AR), Coup transcription factors 1 and 2 (Coup TF's), and Sox 14 are expressed throughout the entire nucleus (red). Steroidogenic factor-1 delineates the more dorsal and central regions of the nucleus (blue). Estrogen receptor α (ERα) and islet-1 are expressed in the ventrolateral quadrant of the nucleus (green), and nkx2.1 is expressed in the lateral region of the nucleus (orange). In panel B, brain derived neurotrophic factor (BDNF), pituitary adenylate cyclase-activating polypeptide (PACAP), and slit3 are expressed throughout the entire nucleus. Neuronal nitric oxide synthase (nNOS), somatostatin (SST), enkephalin (Enkeph), and cholecystokinin (CCK) are expressed in the ventrolateral region (green) with nNOS reaching more dorsolaterally through the nucleus (orange). Panel C depicts various membrane receptors and their expression patterns in the VMH. Cannabinoid receptor-1 (CB-1) is expressed throughout the entire nucleus (red) while the leptin receptor delineates the dorsomedial portion (blue) and the oxytocin receptor (OTR) and the growth hormone secretagogue receptor (GHSR) are expressed in the ventrolateral region (green). In panel D, the expression patterns of GABA and its receptors are illustrated. GAD 65/67 and GABA is made and expressed in fibers that surround the VMH. $GABA_A$ and $GABA_B$ receptors are found within the nucleus. $GABA_B$ receptor subunits are expressed throughout the entire nucleus (red). There are $GABA_A$ receptor subunits expressed throughout the entire nucleus as well, however, each subunit has distinct expression patterns. $GABA_A$ receptor subunit α3 is expressed throughout most of the dorsomedial region (blue), $GABA_A$ receptor subunit α5 is localized to the central region (orange), and the $GABA_A$ receptor subunits β2, β3, γ3 are expressed in the most ventrolateral portion (green).

3. Neurogenesis

The process of developing a functional nucleus begins with the "birth" or terminal mitosis of neurons that populate the region. Based on ^{3}H-thymidine incorporation studies, cells in the VMH derive primarily from precursors in the proliferative zone surrounding the lower portion of the third ventricle dorsal to the arcuate nucleus (Altman and Bayer, 1986). Neurons that populate the VMH are born between E10 and E15 in mice, E13 to E17 in rats, and around E30 in the primate (Shimada and Nakamura, 1973; Tran et al., 2003; van Eerdenburg and Rakic, 1994). Despite having been shown to be important in proliferation and development of adrenals and gonads (Bland et al., 2000), it is unclear whether SF-1 also has a role in neurogenesis within the hypothalamus. In one report, SF-1 was not seen in BrdU immunoreactive cells that were mitotically active along the proliferative zone (Tran et al., 2003). In contrast, another study has shown that SF-1 is expressed in cells of the diencephalon as early as E9.5, when virtually all cells are mitotically active (Ikeda et al., 2001). One group generating the Ftz-F1 (SF-1) KO mouse characterized it to have a thicker ependymal layer, with many immature neurons, in the lower portion of the third ventricle (Shinoda et al., 1995). BrdU data from a second SF-1 knockout mouse indicates that SF-1 expression does not affect the number of neurons generated along the proliferative zone between E11 and E13 (Davis et al., 2004a), suggesting that SF-1 expression does not have a role in neurogenesis. Nonetheless, as discussed below, the time of final mitotic division likely plays a role in the susceptibility to disruption by selective factors.

4. Migration

Following neuronal divisions along the proliferative zone, cells must migrate to form the VMH. BrdU studies in the mouse have shown that cells of the VMH may undergo final mitotic divisions as early as E10, while the earliest sign of cytoarchitectonic boundaries

are not seen until E16-17. Using Nissl stains, the VMH begins to appear as a distinct oval shaped collection of cells on either side of the third ventricle around E18-E19 in rats (Coggeshall, 1964; Hyyppa, 1969), E16-E17 in mice (Schambra et al., 1991; Tobet et al., 1999), and gestational weeks 9-15 in the human (Koutcherov et al., 2002). The apparent organization of cells into a "nuclear structure" involves both the arrival and arrangement of cells, and the development of surrounding fibers that cause the nucleus to appear more densely cellular than the surrounding region. Interestingly, cells identified by phenotype (e.g., ERα) can be found in their correct positions before the boundaries of the VMH can be discerned by Nissl stains (Tobet et al., 1999).

Neurons migrate radially away from ventricular zones guided by radial glial processes and tangential to such fibers, often along neuronal processes (Fig. 4) (Rakic et al., 1994). Based on cortical studies examining the migration patterns of interneurons, radial migration can also occur in the opposite direction as cells move towards the proliferative zone (Hevner et al., 2004). Radial glial fibers are present for both cortical and hypothalamic cell migrations and likely act as guides for migrating neurons throughout the brain. In particular for the VMH, radial glial fibers extend from the third ventricle to the surface of the brain in a dorsomedial to ventrolateral direction. There is evidence for both radial (medial-lateral) and tangential (dorsal-ventral) movements in the region of the developing mouse VMH. The hypothesized pattern of migration is from dorsomedial to ventrolateral (Altman and Bayer, 1986) matching the pattern of radial glial fibers known to cross the VMH (Fig. 4) (Dellovade et al., 2001; Levitt and Rakic, 1980; Tobet and Fox, 1989). In contrast to the inside-out pattern of the cortex, the earliest born cells in the hypothalamus migrate the farthest from the ventricle.

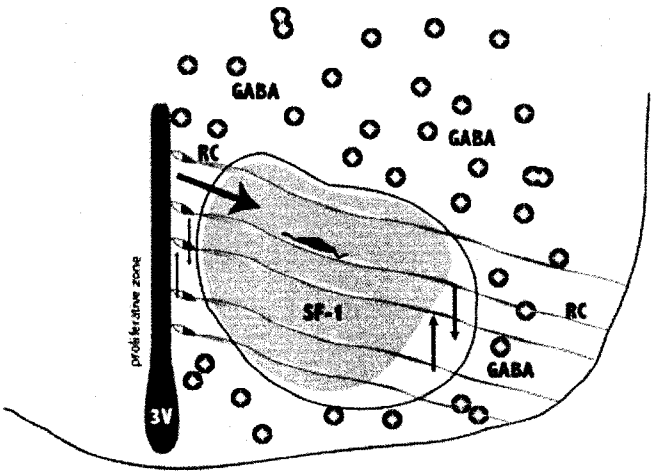

Fig. 4. Schematic representation showing migration from the proliferative zone along the third ventricle (3V) to fill the VMH. Cells migrate along fibers of radial cells (RC) which have their cell bodies located adjacent to the third ventricle and long processes that extend to the pial surface of the brain. SF-1 and GABA are both important factors in this migratory process with SF-1 acting intrinsically within the cells of the VMH and GABA acting extrinsically as a signaling molecule to migrating cells. Migration occurs dorsal/ventral close to the ventricle, in a radial pattern throughout most of the VMH, and dorsal/ventral in the lateral edges of the nucleus (as indicated by the arrows).

4.1 Methods of visualizing migration from the proliferative zone

The migration of neurons from the ventricular zone to the region of the VMH requires cues to help determine direction, speed, and boundaries. Expression of certain genes in development is important in setting up boundaries for migrating neurons (Rubenstein and Rakic, 1999). Three general categories of factors guide neurons to their final positions. Cell surface molecules that attach to the extracellular matrix; cell – cell communication through cell surface molecules; and secreted factors such as chemotropic and chemokinetic factors. Methods involving transgenic mice with cells fluorescently labeled using promoter-driven fluorescent proteins have been created that may help elucidate these cues and their influences on migrating cells (Bless et al., 2005; Dellovade et al., 2001; Henderson et al., 1999). Fig. 5 illustrates one example of using video microscopy techniques to examine cell movements in and around the developing

VMH. Mice with the SF-1 promoter driving enhanced green fluorescent protein (SF-1/eGFP) (Stallings et al., 2002) and mice with the Thy-1 promoter driving yellow fluorescent protein YFP (Thy-1/YFP) (Feng et al., 2000) expression both provide superior ways to visualize the migration of neurons within the hypothalamus (Tobet et al., 2003). Thy-1 is an adhesion protein expressed during development that is evident in cells of the hypothalamus as early as E13. Discussed below are some factors that are known to be involved in the process of cell migration in the region of the VMH.

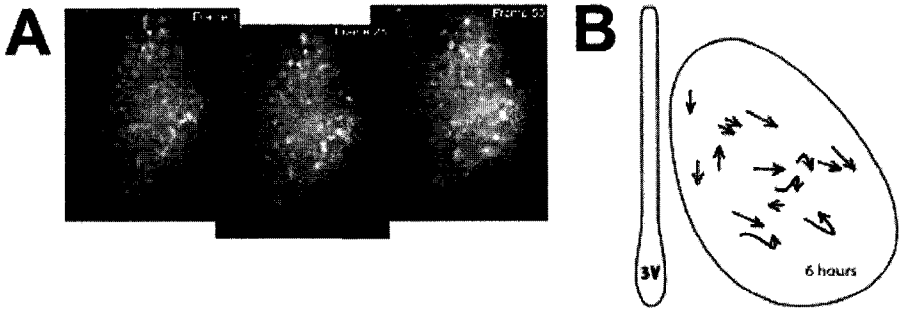

Fig. 5. Live video microscopy of an E14 slice from a mouse expressing YFP in Thy-1 positive cells (A). Three representative frames show the labeling of individual neurons in the region of the developing VMH. Panel B contains a schematic diagram showing the movements of 14 individual cells in 3 different video sequences over the course of 6 hours. As illustrated in the schematic diagram, some cells appear to be moving dorsal/ventral (particularly those in more medial locations) while others are moving in the direction of radial glial fibers. It is common for the cells to turn during the migration process.

4.2 Role of SF-1 in migration

SF-1 exerts a large influence on VMH development by influencing the positions of cells in the region of the VMH, presumably by influencing the movements of migrating cells. Interestingly, some cells born relatively late (E13) are more affected by a loss of SF-1 than cells born earlier (E11) based on a change in the position of BrdU positive cells within the VMH in SF-1 knockout mice (Davis et al., 2004a). There are clearly exceptions to this observation; in the SF-1 knockout mouse ERα cells are unusually laterally placed

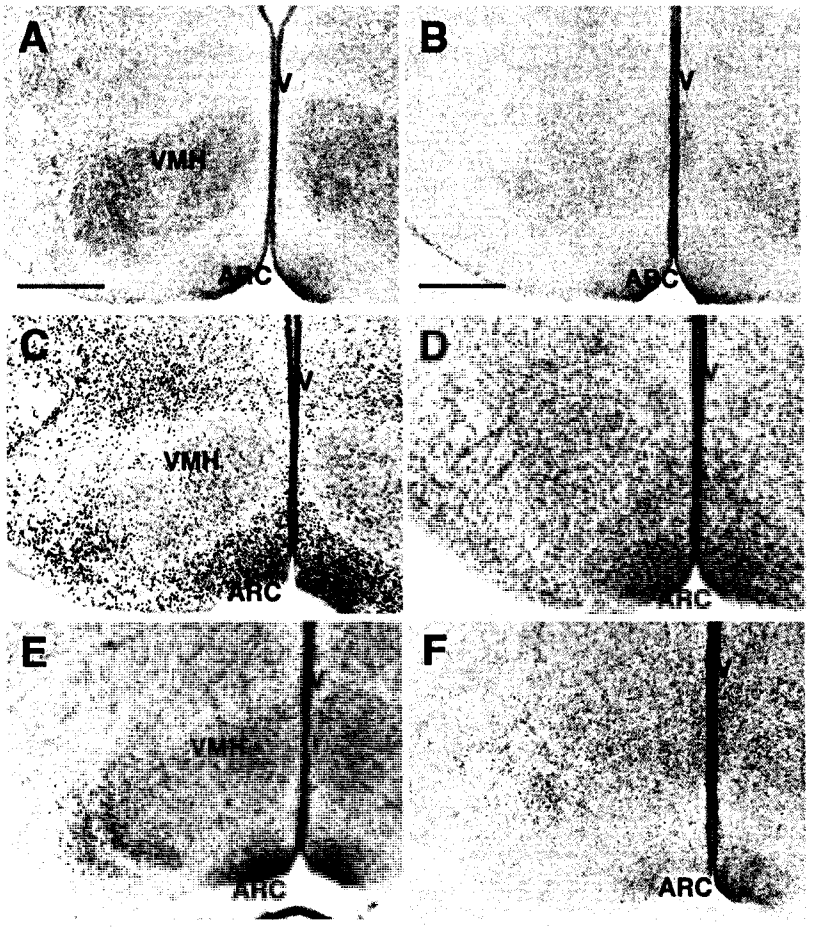

Fig. 6. Nissl stained coronal sections in the region of the VMH: wild-type (A,C,E) versus SF-1 knockout (B,D,F) mice at P0. In panel B, the image from the knockout animal does not have distinct boundaries delineating the VMH as seen in a wild type mouse (A). Nissl conterstained sections immunoreacted for isl-1 shows it's normally expression pattern in cells surrounding the VMH and within the ventrolateral region of the VMH (C). However, in the SF-1 knockout mouse expression is seen throughout the entire region of the VMH with more cells remaining closer to the ventricle (D). Nkx2.1 shows a similar change in expression patterns. The wild type mouse has Nkx2.1 expression in the ventrolateral region of the VMH (E), however this expression pattern is more medial and dorsal in the knockout (F). Sections taken for counterstain are from a previous publication where additional details can be found (Davis et al., 2004a).

and born relatively early (Dellovade et al., 2000). The SF-1 knockout mouse has been an important tool to look at the effect of SF-1 on cell positioning. Groups of phenotypically identified cells are "misplaced" or have an increased scatter pattern in SF-1 null mice. These misplaced cell types include isl-1, nkx2.1 (Davis et al., 2004a), estrogen receptor α, NPY, and galanin positive cells (Dellovade et al., 2000) (Fig. 6). In

addition, a transgenic mouse with a fluorescent transgene (eGFP) driven by the SF-1 promoter was crossed into the SF-1 knockout line resulting in mice that do not express SF-1, yet the cells that would normally express SF-1 fluoresce green. Immunocytochemistry for eGFP+ cells on these animals shows that cells that would normally express SF-1 have an altered positional fate as well as the other phenotypes listed above (Davis et al., 2004a).

4.3 Role of GABA signaling in migration

Several neurotransmitters are proposed to act as neurotrophic factors or morphogens in various brain regions (Lauder, 1993; Nguyen et al., 2001), including GABA, serotonin, dopamine, and endogenous opioids. In the region of the VMH, the distribution of GABAergic elements may play an important role in the arrangement of the cytoarchitecture (Tobet et al., 1999). Four lines of evidence suggest that GABA plays a role in the embryonic differentiation of at least a subset of the cells in the VMH. GABA is synthesized in positions to provide potential boundary information for the embryonic VMH (Fig. 3) (Tobet et al., 1999). Disrupting the SF-1 gene disrupts glutamic acid decarboxylase (GAD)/GABA expression prior to VMH organization (Dellovade et al., 2000). Activation of $GABA_A$ receptors affects cell movements and the distribution of identified cells in the region of the VMH (Dellovade et al., 2001). Finally, activation of $GABA_B$ receptors decreases cell movements in the region of the VMH (Davis et al., 2002). Although the specifics of $GABA_A$ and $GABA_B$ receptor actions may differ, similarly strong indications of a morphogenetic role for GABA have been well characterized in the developing cerebral cortex (Behar et al., 1996; Behar et al., 2000; Behar et al., 1998; Behar et al., 2001). GABA may influence cell positioning by affecting the guidance of cells by neuronal (Bless et al., 2000) or glial (Dellovade et al., 2001) fibers. Manipulation of $GABA_A$ receptor signaling altered the orientation of cell movements (Dellovade et al.,

2001), whereas $GABA_B$ receptor signaling decreased the rate of cell movement (Davis et al., 2002). Since the two receptors may be differentially sensitive to GABA levels (Johnson and North, 1992), the interaction of the two mechanisms may be spatially regulated by the locations of different concentrations of GABA (e.g., high at the nuclear boundary). Studies are underway to determine whether $GABA_A$ and $GABA_B$ receptors act synergistically, antagonistically, or independently to influence movements in the developing VMH. Radial migration occurs throughout the nucleus, however, a neuron can detach from a radial fiber and move tangentially to a specific location along the lateral and medial edges of the nucleus. Another possible role for GABA in migration is through this interaction between neurons and fibers.

4.4 Other factors in migration – cell adhesion

A number of other factors influence the migration of neurons within the hypothalamus, including those that influence early aggregation of progenitor cells, migration, and differentiation. Progenitor cells possess region-specific adhesive properties at early stages of embryogenesis (Krushel and van der Kooy, 1993). A correlation has been made between specific cadherin expression in prosomeres of the diencephalon with the nuclear fate of cells expressing those adhesion molecules (Redies et al., 2000; Yoon et al., 2000). Specifically, cadherin-11 (Kimura et al., 1996b), NRX1, and PCDHα (Mungenast and Ojeda, 2005) transcripts are expressed in the developing mouse brain and are localized to the hypothalamus. N-cadherin, a calcium- dependent adhesion factor is expressed along the apical surface of the ventricular zone in the E14 mouse hypothalamus. Transplantation experiments looking at the isolation of progenitors dissected from different prosomeres were used to study intrinsic prosomeric identity. Forced expression of R-cadherin (another calcium-dependent adhesion molecule), in these transplanted diencephalic progenitors helps cells to integrate where this cadherin

is expressed (McCarthy et al., 2001). Adhesion molecules are important for movement along radial glial cells as well. Factors influencing adhesion between neuron and glia are key elements in establishing positioning within a nucleus. For example, the gene and product Disabled-1 causes adherence of neurons to glia and a mutation in this gene could prevent a neuron from dissociating from a glial fiber and moving to a certain position within the cortex (Sanada et al., 2004). Certain genes are also involved in reducing the adhesive properties of neurons to glia. At the cortical plate, sparc-like 1 causes the termination of neuronal migration by causing neurons to lose adhesiveness to radial glial fibers (Gongidi et al., 2004). Neurofascins are also involved in neuronal to glia adhesion. They are expressed by neurons and glia and can play roles in both inhibition and promotion of neuronal cell adhesion (Koticha et al., 2005). The cytoskeleton plays a critical mechanical role in the migration of neurons. Migrating neurons have a characteristic shape including a leading process that can extend relatively long distances away from the cell soma (Hatten, 2002). Directional movement requires the extension of the leading process and the translocation of the cell soma. Cytoskeletal rearrangement is involved in this movement and in particular myosin II plays a key role in translocation of the neuronal cell body and the release of the rear of the neuron (Schaar and McConnell, 2005).

5. Programmed cell death

In the developing brain, programmed cell death plays a major role in the organization of brain structures (Gordon, 1995). Almost 10 years ago it was realized that there may be significantly more cell death during normal development than previously appreciated (Blaschke et al., 1996). Subsequent experiments revealed significant cell death in regions of the hypothalamus that are sexually dimorphic (Arai et al., 1996; Davis et al., 1996; McCarthy et al., 1997). Sex differences in cell death have emerged as a central

component of many theories of brain sexual differentiation (Forger et al., 2004). There is contrasting information on cell death in the region of the VMH that may be due in part to specific strain differences in rats. In Sprague Dawley rats, there was very little apoptosis during postnatal development. As the nucleus increased in size from PN2 – PN12, the amount of cell death decreased (Chung et al.). Cell death was also examined in the VMH of Wistar rats at postnatal ages. There was a greater incidence of cell death observed in these rats at birth, and again a decrease in the number of apoptotic cells at later postnatal ages.

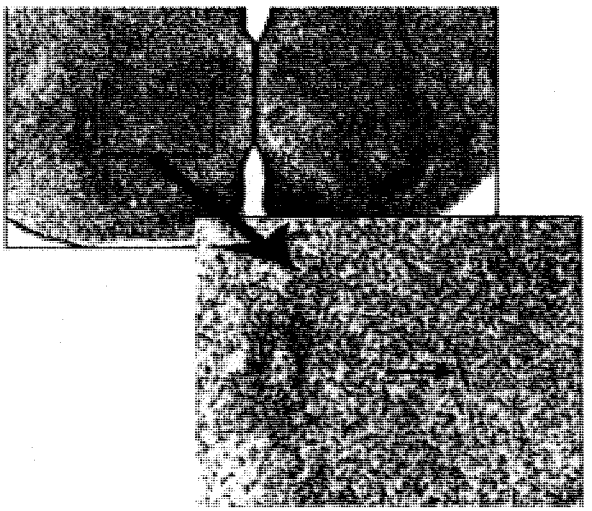

Fig. 7. The image is taken from a 50μm coronal tissue section of a P0 C57 mouse processed for ir activated caspase 3 (antisera obtained from Chemicon Inc.) and stained with a 0.5% basic fuschin counterstain to visualize the VMH. The blue arrow indicates an activated caspase-3 ir cell at high magnification. Few activated caspase-3 immunoreactive cells are in the region of the VMH suggesting that cell death plays a minor role in this stage of development.

Based on the locations of dying cells, there does not appear to be a major role for cell death in the emergence of the VMH and there is no more cell death at the periphery of the forming VMH than in any particular region of the VMH. Our own examination of cell death in the VMH also led us to conclude that cell death does not play a major part in the emergence of the VMH. Pyknotic cells (an indicator of apoptosis) were counted in VMH sections and no more than 4 cells were found per each 50μm thick section (Davis et al.,

2004a). Caspase3 is a widely used marker of apoptotic cells and in the region of the VMH at P0; few cells express this marker in mice (Fig. 7), again suggesting only a minor role for programmed cell death in this region.

5.1 Factors that may play a role in cell death/ survival

In conjunction with cell death, cell survival is also an important part of the development of any organized brain structure. The discovery of nerve growth factor in the late 1950's by Rita Levi-Montelcini led to the discovery of a large family of growth factors important for cell survival and growth. One of these growth factors – brain derived growth factor (BDNF) – has a strong expression pattern in the developing mouse VMH (Fig. 2). In the rat, BDNF expression is high from birth through P6, followed by a rapid decrease in BDNF expression from P6 through adulthood (Sugiyama et al., 2003). In the SF-1 knockout mice, there is an apparent decrease in BDNF expression suggesting that SF-1 regulates the expression of this neurotrophic factor (Tran et al., 2003). These changes in BDNF expression in the developing brain indicate that it may play a role in cell differentiation or function in the developing VMH. BDNF has been shown to play a role in the migration of cerebellar granule cells as well as some cortical neurons thus demonstrating potential chemokinetic and chemotactic roles for BDNF (Behar et al., 1997; Borghesani et al., 2002). However, when brains from BDNF knockout mice generously provided by Dr. R.A. Segal (Harvard University, Boston, MA) were examined for changes in VMH cell positioning, there was no obvious alterations in cell positions suggesting that the role BDNF plays in the development of the VMH relates to physiological function(s) and does not involve cell migration (S.A. Tobet, unpublished observations).

6. Connections

The major afferent connections to the VMH include the preoptic area, thalamic and epithalamic areas, amygdala, and dorsal midbrain including the medial central gray (Canteras et al., 1994; Fahrbach et al., 1989). The major efferent connections from the VMH are to the amygdala, medial preoptic area, anterior hypothalamus, bed nucleus of the stria terminalis (BST), central gray, zona incerta, and the peripeduncular nucleus (Saper et al., 1976) (Fig. 8). These projections have been examined in the adult mouse, rat, and guinea pig; however, not much is known about when these projections are established. While the VMH is established as a nucleus by E17, the earliest studies on connections are during the first postnatal week. Retrograde labeling shows that the afferent connections from the amygdala to the VMH are apparent as early as 1 week after birth (Choi et al., 2005). A detailed study has analyzed projections from specific subregions within the VMH of adult rats (Canteras et al., 1994). Neurons containing steroid hormone receptors in the ventrolateral VMH project to the dorsal midbrain (Morrell and Pfaff, 1982), and 30% of the neurons in the ventrolateral region expressing both estrogen receptor α and somatostatin project to the medial central gray in guinea pig (Dufourny and Warembourg, 2001). Most afferent connections to the VMH are to the ventolateral region of the nucleus, the area with cells expressing estrogen receptors (Fahrbach et al., 1989). Projections from the VMH are known to mediate various functions associated with this region. The ventrolateral region, with its large population of steroid hormone receptors is involved in sexual behavior. Projections to the medial central gray and periaqueductal gray have been shown to be involved in the pathway leading to the lordosis response in female rats (Flanagan-Cato et al., 2001). It has also been shown that sites of the hypothalamus containing estrogen receptors are likely to project to other estrogen receptor-immunoreactive sites in the female guinea pig (Turcotte and Blaustein, 1999).While the ventrolateral population of cells sends out

projections important in sexual behavior, the dorsomedial subdivision may be important for feeding behavior. NPY positive fibers travel through the dorsomedial and central portions of the nucleus and the leptin receptor is located in this region as well (Fig. 3). Although not much is known about events in development contributing to obesity in adulthood, we hypothesize that the development of the VMH and positioning of individual cell types is important in establishing connections related to feeding behavior and obesity. A major goal for future studies is to define the molecular keys for developing VMH connections and the behaviors resulting from the formation of these connections. It also will be very interesting to determine the degree to which results obtained in the VMH can be generalized to the formation of other nuclear structures. For example, the transcription factor SIM1 is essential both for the formation of the paraventricular nucleus (Michaud et al., 1998a) and for the regulation of behaviors associated with the PVN (Michaud et al., 2001). Do other transcription factors similarly play "master" roles in the organization of hypothalamic nuclei such as the DMH or in the development of discrete nuclei in structures such as the thalamus?

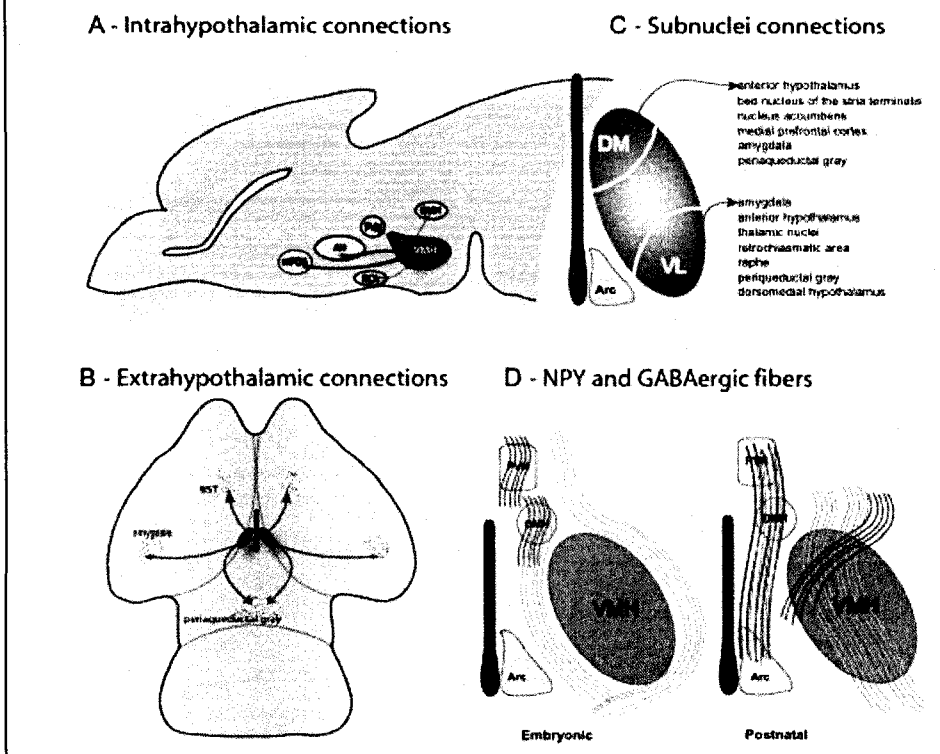

Fig. 8. The neurons of the VMH send out axonal connections to other brain nuclei and regions both within the hypothalamus (panel A) and outside of it (panel B). Many neurons connect with the anterior hypothalamus (AH) and the medial preoptic area (mPOA) within the hypothalamus, while a smaller subset of neurons sends connections to the dorsal medial hypothalamus (DMH), the paraventricular nucleus (PVN) and the retrochiasmatic area (RCA) that surrounds the suprachiasmatic nucleus. The major extrahypothalamic VMH connections are to the amygdala (B), bed nucleus of the stria terminalis (BST), and the periaqueductal gray. The images show the third ventricle in brown, nuclear groups in shades of yellow, and the VMH is shown in blue. The VMH is comprised of subnuclear groups including the dorsomedial (DM) and ventrolateral (VL) which project to specific regions as they mediate various functions and behaviors (panel C). The VMH is not only made up of neurons projecting and receiving connections, but of fiber pathways surrounding and moving through the VMH (panel D). In the developing rat VMH, NPY immunoreactive fibers (blue) are moving through the PVN and the DMH while GABAergic fibers (orange) are surrounding the region of the VMH. By P10 NPY and GABAergic fibers are extending from the arcuate nucleus (Arc) to the PVN. NPY and GABAergic fibers have also moved into the region of the VMH with the NPY fibers restricted the dorsomedial and central parts of the nucleus.

7. Conclusion

There is a wealth of information regarding the structure and function of various hypothalamic nuclei; however, there is much less known about their development and factors that may be involved. The VMH provides a model for hypothalamic development and for gaining insight regarding the mechanisms and factors that are involved in the development of a cell group. The VMH appears as a well defined cell group just days before birth in the rodent, and reaching this stage requires the proliferation, migration, and survival of neurons that make up the nucleus. Neurons originate along the proliferative zone, adjacent to the third ventricle, and migrate along radial glial cells to final positions within the VMH. The VMH is a heterogeneous nucleus, made up of anatomical subnuclei with specific molecular identities. A number of factors are likely to be important in the positioning of cells within the VMH, including SF-1 acting intrinsic to VMH neurons and GABA acting extrinsically. We hypothesize that SF-1 influences migrating neurons by affecting the transcription of various genes needed for movement or guidance of these neurons. For example, SF-1 may influence the transcription of Slit proteins, which are expressed in the VMH and are known to be involved in growth cone or cell guidance and/or movement. We also hypothesize that GABA is affecting migration through two possible mechanisms. GABA binds to its receptors, which can indirectly influence adhesion of cells to radial glial fibers through calcium dependent mechanisms. This could cause a change in speed or position of these cells as their ability to adhere to a radial glial fiber decreases. Another possible mechanism for the action of GABA is through an effect on cytoskeletal proteins important in cell movement. See Figure 3 for a schematic of the expression pattern of these proteins. Cell positioning within the VMH is extremely important in maintaining a functional nucleus, as a cell that has reached an altered position may not attain its correct phenotypic identity and become properly connected. Once in place, the phenotypic differentiation of neurons is

crucial for maintaining function. Afferent and efferent connections mediate behaviors associated with the VMH including female sexual behavior, feeding behavior, cardiovascular function, and pain sensitivity. Understanding the development of the VMH, as well as other hypothalamic cell groups, is a critical piece to understanding its role in behaviors as well as how these behaviors can be altered. There is still much to discover about the factors and mechanisms involved in guiding the development of hypothalamic nuclei.

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Chapter 3

$GABA_B$ Receptors Role in Cell Migration and Positioning within the Ventromedial Nucleus of the Hypothalamus

Abstract

The ventromedial (VMN) and arcuate (ARC) nuclei of the hypothalamus are bilateral nuclear groups at the base of the hypothalamus that are organized through the aggregation of neurons born along the third ventricle that migrate laterally. During development, GABAergic neurons and fibers surround the forming (or primordial) VMN while neurons containing GABA receptors are found within the boundaries of the emerging nucleus. To investigate the role that $GABA_B$ receptors play in establishing the VMN, Thy-1 YFP mice were utilized for live video microscopy studies. The Thy-1 promoter drives YFP expression in regions of the hypothalamus during development. Administration of the $GABA_B$ receptor antagonist saclofen and the $GABA_A$ receptor antagonist bicuculline selectively increased the rate of VMN cell movement in slices placed in vitro at embryonic day 14, when cells that form both the ARC and VMN are migrating away from the proliferative zone surrounding the third ventricle. To further test the role of $GABA_B$ receptors in VMN development, $GABA_B$ receptor knockout mice were used to examine changes in the positions of phenotypically identified cells within the VMN. Cells containing immunoreactive estrogen receptors (ER)α were located in the ventrolateral quadrant of the wild type VMN. In $GABA_BR1$ knockout mice, these ERα positive neurons were located in more dorsal positions at postnatal day (P)0 and P4. We conclude that GABA alters cell migration and its effect on final cell positioning may lead

to changes in the circuitry and connections within specific nuclei of the developing hypothalamus.

Introduction

The ventromedial nucleus of the hypothalamus (VMN) first appears using Nissl stains as a bilateral cell group at the base of the diencephalon around embryonic day 16/17 (E16/17) in mice (McClellan et al., 2006). The heterogeneity of the VMN contributes to the many roles it plays in neuroendocrine function. These roles include influencing female sexual behavior, feeding behavior, anxiety/ defensive behavior, and pain (Canteras et al., 1994; Dielenberg and McGregor, 2001; King, 2006). The VMN is loosely categorized into three main regions: dorsomedial, central, and ventrolateral (Saper et al., 1976; Van Houten and Brawer, 1978). The dorsomedial and central regions are characterized by the expression of the transcription factor steroidogenic factor-1 (SF-1), and the ventrolateral region can be characterized by the expression of ERα (Dellovade et al., 2000; Simerly et al., 1990). SF-1 is one critical transcription factor in VMN development as it plays a role in establishing the cytoarchitecture of the nucleus through terminal differentiation (Tran et al., 2003) and distribution of neuronal phenotypes (Davis et al., 2004a; Dellovade et al., 2000). Although SF-1 is important in VMN development, our prior work and work of others suggests that other factors, in particular, gamma-aminobutyric acid (GABA) are also likely to be involved in determining the boundaries of the nucleus by influencing the movement characteristics of migrating neurons.

The neurotransmitter GABA has an interesting relationship with the development of the VMN (Tobet et al., 1999). During early stages of development, GABA is synthesized in positions that could provide potential boundary information for the embryonic VMN.

GABAergic neurons and fibers surround the embryonic VMN, and towards the end of gestation in mice, GABAergic fibers begin to infiltrate interior regions of the VMN (Tobet et al., 1999). In contrast to the late gestational in-growth of fibers, subunits for $GABA_A$ (Dellovade et al., 2001), and $GABA_B$ (Davis et al., 2002) receptors are expressed in neurons within the region of the VMN as early as E13 and throughout adulthood. In addition, physiological analyses have been performed as early as E18 and indicate the presence of functional receptors in the developing mediobasal hypothalamus of rats (Obrietan and van den Pol, 1995; Obrietan and van den Pol, 1998).

In addition to its role as the major inhibitory neurotransmitter in the adult CNS, GABA is also important in many developmental processes, including cell proliferation (LaMantia, 1995) and neuronal migration (Behar et al., 1996; Behar et al., 1998; Manent and Represa, 2007). GABA's influence on migration can be mediated through either or both its A and B receptors. In cortical migration, $GABA_A$ and $GABA_B$ receptors play a role in the formation of cortical layers (Behar et al., 1998). Data to date indicate that the ability of GABA to influence neuronal migration within the VMN is mediated by both ionotropic $GABA_A$ (Dellovade et al., 2001) and metabotropic $GABA_B$ receptors (Davis et al., 2002). The $GABA_A$ receptor agonist muscimol caused a decrease in the percent of neurons moving within the region of the developing VMN. The addition of baclofen, a $GABA_B$ receptor agonist, to live tissue slices also caused a dose-dependent decrease in the rate of motion of cells in the region of the VMN (Davis et al., 2002). Baclofen administration did not change the probability of cells moving nor did it have an effect on the angle of cell movement. Based on the Nissl stained gross cytoarchitecture, baclofen did not influence the ability of the VMN to form.

The current study further examines the role of $GABA_B$ receptors in the development of the embryonic and early postnatal murine VMN and compares the role of these receptors on the development of the neighboring arcuate nucleus (ARC). Mice in which the Thy-1 promoter drives neuron-selective yellow fluorescent protein (YFP) expression (Feng et al., 2000), were utilized for live video microscopy studies in vitro to evaluate the potential role of endogenous GABA acting at GABA receptors on the movement of one population of migrating neurons (Thy-1/YFP expressing neurons) by using GABA receptor antagonists. These mice were used as a tool to follow the movement patterns of fluorescently labeled cells in the regions of the VMN and ARC. It is unknown as to why YFP expression is found in a subset of VMN and ARC neurons at embryonic ages, but the limited pattern of expression is likely due to the insertion site of the transgene, the number of copies incorporated into each line, or the interactions of the transgene elements with flanking DNA (Feng et al., 2000). $GABA_B$ receptor knockout mice (Prosser et al., 2001) were utilized to determine the dependence of VMN formation on $GABA_B$ signaling during early development. Immunocytochemistry and in situ hybridization were used to identify cell phenotypes for ERα, BDNF (brain derived neurotrophic factor), SF-1, and specific $GABA_A$ receptor subunits to determine potential differences in cell position or expression in mice without $GABA_B$ receptor signaling.

Materials and Methods

Animals

Two lines of transgenic mice were used for the experiments in this study. $GABA_BR1$ heterozygous breeding pairs obtained from GlaxoSmithKline (Middlesex, UK) were used to generate knockout, heterozygous, and wild type mice at multiple developmental ages. Mice with disruption of $GABA_B$ receptor signaling were generated on a C57BL/6 background through the insertion of a gene encoding β-galactosidase in the coding

region of the R1 subunit of the $GABA_B$ receptor (Prosser et al., 2001). Another line of transgenic mice obtained from The Jackson Laboratory (*B6.Cg-Tg(Thy1-YFP)16Jrs/J;* Bar Harbor, Maine) were created on a C57BL/6 background with the Thy-1 promoter driving YFP expression (Feng et al., 2000). The Thy-1 promoter in this transgenic line drives neuronal expression in the brain, including the ARC and VMN, in a spatially and temporally regulated manner during development (Knoll et al., 2007; Tobet et al., 2003). $GABA_BR1$ heterozygote mice were crossed with Thy-1/YFP mice to begin to generate a line of $GABA_BR1$ knockout and heterozygote mice that also contain fluorescently labeled YFP neurons. Animals were mated overnight and females were checked for vaginal plugs the following morning. The day of plug was designated as E0 and mice were taken at ages E15, E17, P0 (counted as day of birth - occurring at E19), and P4. Pregnant dams were anesthetized using ketamine (80mg/Kg) and xylazine (8mg/Kg) and embryos were removed by Cesarean section one at a time. Crown rump lengths were measured to verify developmental age, weights were taken for comparison, and tissue was taken for genotyping by PCR. P0 and P4 pups were anesthetized on ice and pups were perfused transcardially with either 4mL (E17) or 5mL (P0 and P4) of 4% paraformaldehyde. Heads were postfixed overnight in 4% paraformaldehyde and changed into 0.1M phosphate buffer (PB) the following day. Heads were stored in 0.1M PB at 4°C until used for immunocytochemistry or in situ hybridization. All experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Colorado State University Animal Care and Use Committee.

Genotyping

Tail DNA was extracted using a base extraction method – incubation at 95°C with 0.05M NaOH for 20 minutes, 10 minutes at room temperature, followed by addition of a Tris NaOH solution (pH 8.0) and stored at -20°C. Mice carrying the $GABA_BR1$ (Genbank

accession number NW001470; (Kaupmann et al., 1997)) knockout allele were genotyped using standard Taq polymerase PCR kit (Qiagen; Valencia, CA). The PCR cycling conditions consisted of 20 seconds of denaturation at 94°C followed by 20 seconds of annealing at 56°C and 1 minute of extension at 72°C. This was repeated for 35 cycles. $GABA_BR1$ primer sets used consisted of a KO insert forward primer (CGCCTTCTTGACGAGTTCT), a WT-specific forward primer (CTCCCGAGAGTGACTGGTT) and a common reverse primer (GCCCTCTTGCCTCTCTAAA). Animals were designated as wild type, heterozygous, or knockouts. Wild type and heterozygous animals had no detectable differences on any measure and were grouped together as controls. For video microscopy studies, mice were either heterozygous or homozygous for the Thy1-YFP allele. PCR cycling conditions for YFP genotyping consisted of 30 seconds of denaturation at 94°C followed by 1 minute of annealing at 56°C and 1 minute of extension at 72°C. This was repeated for 35 cycles. YFP primer sets included a forward primer (AAGTTCATCTGCACCACCG) and a reverse primer (TCCTTGAAGAAGATGGTGCG). Sex determination was done using PCR for the Y chromosome encoded *Sry* gene as described previously (Luo et al., 1994).

Immunocytochemistry and In situ hybridization

For immunocytochemical experiments brains were dissected out of the head, embedded in 5% agarose, and cut into 60µm thick sections using a vibrating microtome (VT1000S; Leica Microsystems, Wetzlar, Germany). Alternating sections were collected in 0.05M phosphate buffered saline (PBS) and incubated with primary antisera. Rabbit polyclonal antisera directed against ERα was obtained from Upstate Biotechnology (C1355; Charlottesville, VA) and was diluted to 1/5000 for brightfield detection and 1/1000 for epifluorescence. Other rabbit polyclonal antibodies obtained include SF-1 (graciously

provided by Dr. Ken Morohashi; diluted 1/1000), $GABA_A$ receptor subunit β3 (graciously provided by W. Sieghart; (Sperk et al., 1997); 0.045μg/mL), $GABA_A$ receptor subunit α1 (Phosphosolutions, Aurora, CO; diluted 1/200), and green fluorescent protein (GFP; Molecular Probes/Invitrogen, Carlsbad, CA; diluted 1/50,000). A chicken polyclonal GFP antibody was also used for dual label epifluorescence (ckGFP; Chemicon International, Temecula, CA; diluted 1/1000). Guinea Pig polyclonal antisera directed against $GABA_BR1$ (GP311) was generously provided by Dr. Marta Margeta-Mitrovic (Margeta-Mitrovic et al., 1999) and was used for dual label with GFP and ERα. Radial Cell (RC)2 antibody (diluted 1/3) that is selective for a protein in radial glia (Chanas-Sacre et al., 2000) and islet-1 antibody (diluted 1/30) was obtained from the developmental studies hybridoma tissue bank (DSHB; Iowa City, IA). Methods for immunocytochemical procedures using floating tissue sections were reported previously (Davis et al., 2002; Dellovade et al., 2000; Tobet et al., 1996; Tobet et al., 1999). Briefly, sections were washed in 0.05M PBS with 0.1M glycine for 30 minutes followed by a series of rinses with PBS. The sections were then placed in a 0.5% solution of sodium borohydride in PBS, rinsed in PBS, and incubated in a PBS blocking solution containing 5% normal goat serum, 0.3% Triton-X100 (Tx)/PBS and 1%H_2O_2. Primary antibodies were diluted in a PBS buffer containing 1% bovine serum albumin and 0.3%Tx. The sections were then placed in primary antibody for 2-3 nights at 4°C. Following the incubation in primary antibody, all steps were done at room temperature. Sections were rinsed 4 times in a PBS/ 1% normal goat serum solution containing 0.02%Tx, and then incubated in secondary antibody for 2 hours. The secondary antibodies were obtained from Jackson ImmunoResearch Labs (West Grove, PA) and were diluted in a PBS solution containing 1% normal goat serum and 0.32%Tx. Following this incubation the sections were rinsed 4 times in a PBS/ 0.02%Tx solution. For brightfield detection a 1:2500 dilution of

peroxidase conjugated streptavidin in 0.32% Tx/ PBS was placed on the tissue sections for 1 hour. The sections were rinsed 4 times in a Tris buffered solution (TBS; pH 7.5) and incubated in 0.025% diaminobenzidine/ 0.02% Nickel/ 0.02% H_2O_2 solution diluted in TBS. This reaction was run for 5 minutes in each immunocytochemistry experiment and the sections were washed in TBS and stored at 4°C until the tissue was mounted the following day. The controls included looking at an additional $GABA_A$ receptor $\alpha 1$ antibody with corroborating results and antisera preabsorption, as well as incubation of the tissue without primary antibody.

For generation of BDNF riboprobes, plasmids containing the coding sequence for BDNF (provided by Dr. Keith Parker) were linearized using the appropriate restriction enzyme (Pst1 – sense, EcoRV – antisense). The linearized DNA was extracted using a standard phenol chloroform extraction. The linearized DNA was then transcribed and DIG labeled using a Digoxigenin labeling kit (Roche Applied Science, Indianapolis, IN) and the appropriate RNA polymerase (T3 – sense, T7 – antisense). The DIG labeled RNA probes were then precipitated in a solution containing 1µl glycogen (20mg/ml Boehringer Manheim), 2.5µl 4M LiCl, and 75µl cold 100% EtOH. The solution was vortexed briefly, incubated at -80C, washed again with 70% EtOH, left to air dry, and resuspended in DEPC water. An optical density reading was obtained to estimate the amount of probe in solution. For BDNF 0.2ug of the riboprobe was added to 1mL of pre-hybridization buffer for each reaction. Sense probes were generated and used as a control.

For in situ hybridization experiments, tissue was collected using the same methods used for immunocytochemistry but was cut into 100µm sections. The in situ hybridization protocol used was reported previously (Dellovade et al., 2000) and was adapted for using free floating sections from previously published methods (Riddle et al., 1993) using

polyvinyl alcohol in the reaction product step to enhance detection (De Block and Debrouwer, 1993). Briefly, the in situ hybridization protocol involved a series of steps occurring over 4 days that included hybridization, washes, and detection using a non-radioactive digoxigenin system. On day 1, agarose embedded brains were cut into cold DEPC-treated PBS with 12% NaCl and followed by a one hour bleaching step of 6% H_2O_2 in PBT (PBS with 0.1% Tween-20) at room temperature (RT). Sections were then washed with PBT and treated with 10µg/ml Proteinase K diluted in PBT, followed by a wash in 2mg/ml glycine diluted in PBT, rinsed in PBT, then placed in a post-fix solution (4% paraformaldehyde/ 0.2% glutaraldehyde diluted in PBT) for 20 minutes. After PBT rinses, the sections were moved to a prehybridization solution (for 40mls of prehybridization solution: 20ml formamide, 4ml 20X SSC, 0.182ml Yeast tRNA, 2mg heparin, 9.56ml of 1g/10ml solution dextran sulfate, 6.26ml DEPC H_2O). The RNA probes were heated to 85°C for 5 minutes to denature, and riboprobes were then transferred to the vials containing the tissue and prehybridization solution. The sections were left overnight at 60°C to hybridize. The following day the sections were washed with a 50% formamide solution (25% 20X SSC, pH4.5 and 25% MQH_2O) in a 60°C shaking water bath, and then washed in a second solution (50% formamide; 10% 20X SSC, pH4.5; 40% MQH_2O) in a 60°C shaking water bath. The sections were washed at room temperature (RT) in a fresh solution of TBST (1L solution: 8g NaCL, 0.2g KCl, 25mls 1M Tris HCl, 10mls Tween-20, up to 1L MQH_2O). The tissue was placed in a pre-block solution consisting of 10% sheep serum diluted in TBST for at least 1 hour at RT and anti-dig-AP antibody (1:2000) in 1% sheep serum/ TBST was put into each boat and then shaken overnight at 4°C in a humid chamber. The following day, the antibody was removed and the tissue was washed in TBST. Next, the sections were washed 5 times in TBST, rocking at RT, and then left overnight in TBST at 4°C. On the final day, the

sections were washed in NTMT (2% 5M NaCl; 5% 2M Tris, pH 9.5; 5% 1M $MgCl_2$; 1% Tween-20; diluted in MQH_2O), and were incubated in 4 ml of a color detection mix in the dark and monitored every 1-2 hours for signal detection. The NBT/BCIP color detection mix recipe (25ml) consists of 12.5mls 20% polyvinyl alcohol; 12.3mls 200mM Tris, pH 9.5; 0.5mls 5M NaCl; 0.125mls 1M $MgCl_2$; 0.225 mls Levamisol; 0.5mls NBT/BCIP. Once the reaction was determined complete based on high contrast signal over background, the sections were washed in NTMT, and then washed in a post substrate solution (5ml 1M Tris-HCl; 1ml 0.5M EDTA; 4.5g NaCl; 0.5ml MQH_2O). Following final washes in PBT the sections were stored at 4°C until mounted onto glass slides and coverslipped using aqueous mounting medium.

Slice Preparation and Live Video Microscopy

Mice were time mated and fetuses were taken at E14, a developmental age when $GABA_B$ receptors are expressed in the hypothalamus in the region of the developing VMN (Davis et al., 2002). Pregnant mice were deeply anesthetized with ketamine (80mg/Kg) and xylazine (8mg/Kg) and fetuses were taken by Cesarean section one at a time. The brain, with the pituitary and surrounding cartilage attached, was dissected out of the skull. Dissections were done in Krebs buffer (126mM NaCl, 2.5mM KCl, 1.2mM NaH_2PO_4, 1.2mM $MgCl_2$, and 2.5mM $CaCl_2$ and an additional 11mM glucose and 25mM $NaHCO_3$) on ice and tissue was taken for sex determination (Sry PCR) and genotyping ($GABA_B$ PCR). Heads were processed for live slice preparations following previously published methods (Tobet et al., 2003). Briefly, dissections were limited to a maximum of two hours or eight pups to minimize cellular damage or death. Following each dissection, brains were embedded in 8% agarose and cut in a coronal plane at 250μm in Krebs buffer using a vibrating microtome (Leica VT1000S). Slices that contained regions of the hypothalamus that included the VMN were chosen for video microscopy and were

transferred to media (Neurobasal; GIBCO-Invitrogen Corporation, Carlsbad, CA), with 10% L-glutamine, 2% B-27, 1.1% glucose, 2% pen-strep, 2% glutamine and incubated at 36°C with 5% CO_2 for 35 minutes. Following the 35-minute incubation period, each slice was plated onto glass bottom dishes pre-coated by the manufacturer with poly-d-lysine (MatTek; P35G-0-20-C) and coated with a 1:1 dilution of Vitrogen (Cohesion Technologies, Inc., Palo Alto, CA) and water. The slices were put back into the 36°C incubator for up to 1 hour to promote adherence. To ensure that slices would move minimally during video observation, 40µL of a Vitrogen solution (1mL Vitrogen, 125µL 10XMEM, 23µL penicillin/streptomyocin, and 33µL 1M sodium bicarbonate) was placed over each slice. The slices were then placed in the 36°C incubator for 1.5 hours to allow the Vitrogen to polymerize. 1mL of Neurobasal media was pipetted into each dish. The slices were maintained at 36°C and 5% CO_2 until use for video microscopy- as early as the following morning and as late as 3 days post plating.

In preparation for video microscopy, slices were washed 3 times with warm Neurobasal media (GIBCO-Invitrogen), and placed on a heated stage maintained at 37°C with 5% CO_2 and with fresh Neurobasal medium in the dish. All data was collected on either a Nikon TE200 microscope or a Nikon TE2000-U (Nikon USA, Melville, NY) with a 20x plan Apo phase objective. Cells expressing yellow fluorescent protein (YFP) were imaged using Metamorph software (Molecular Devices Inc., Sunnyvale, CA). A digital camera captured a z-stack series of three images at 5µm intervals through the tissue. A set of three images was taken every 5 minutes throughout the duration of the video experiment. At least 1.5 hours of baseline video microscopy was taken before the addition of GABA receptor antagonists, saclofen (10µM-100µM, $GABA_B$; Sigma-Aldrich, St. Louis, MO) or bicuculline (10µM, $GABA_A$; Sigma-Aldrich, St. Louis, MO). The

concentrations used were chosen based on the intent to block GABA signaling through its receptors in live brain slices (Dellovade et al., 2001; Harrison et al., 1990). Once a drug was administered to the slice, an additional 1.5 hours of video was taken to compare treatments.

Imaging and Analysis

For immunocytochemical analysis, each section of tissue that contained a region of the VMN, easily identified based on cell and background density, was labeled as A, B, C, or D; extending from rostral to caudal, respectively, (McClellan et al., 2006). Digital images were taken on an Olympus BH2 microscope with an Insight QE digital camera using Spot Advanced software (Draper UT). Images were taken from the sections labeled B and C, those most centrally located within the nucleus, and then normalized for optimal contrast using Adobe Photoshop software (Version CS for Macintosh). For the analysis of cells containing immunoreactive ERα, images were opened in IP Lab software (Scanalytics Inc. part of BD Biosciences, Rockville, MD) and grids (50μm x 50μm) were placed over the images with the boundaries being the base of the brain and the third ventricle (Davis et al., 2004b). Rows and columns extended from the base of the brain moving dorsally and laterally from the third ventricle. Row 1 was designated as the row closest to the base of the brain with more than 50% of the boxes containing tissue. Areas were determined following standard segmentation of the most densely reactive cellular elements in each image. The area of ERα immunoreactive cells was measured in each box and designated as immunoreactive cells residing in the region of the ARC or VMN. Totals were calculated by region and by row, and statistical significance were determined by ANOVA for genotype x age and using row as a repeated measures using SPSS software (SPSS Inc.; Chicago, IL). Immunoreactive SF-1 and the in situ hybridization signal for BDNF were analyzed as total area within the VMN by one-way

ANOVA for genotype. As there was no evidence for a heterozygote phenotype for these measures the data depicted is control (wild type + heterozygotes) versus homozygous knockout. For dual label fluorescence, images were taken on a Zeiss LSM510 Meta confocal microscope.

Video analysis has been described in detail previously (Bless et al., 2005; Knoll et al., 2007). Video sequences were evaluated for the presence of visibly moving Thy-1 YFP neurons. These images were aligned to normalize for slice movement using Image J software (NIH, Bethesda, MD), and movement analysis was performed using MetaMorph (Molecular Devices, Downington, PA). A fluorescent cell was "tracked" by manually following the center of each cell and recording the position for each time point. The rate, or average speed, was determined by calculating the speed between each frame and taking an average of all speeds. Data was first collected for all moving cells and later each cell was classified as being part of the VMN or ARC. Data for the current study was collected from 20 slices, from 16 litters.

Results

GABA receptor antagonists caused an increase in movement of fluorescently labeled neurons in the region of the VMN.

Previous experiments have shown the effect of the $GABA_B$ receptor agonist, baclofen, on the movement of neurons randomly labeled with the lipophilic carbocyanine dye DiI in the region of the VMN (Davis et al., 2002). For the current study transgenic Thy-1/YFP mice were used as a tool to visualize cell movements in the region of the VMN and ARC. Neurons expressing YFP are found in the embryonic hypothalamus in numbers that make it possible to visualize distinct cells in the VMN and ARC for the purpose of tracking and motion analysis. The subpopulation of ARC and VMN neurons that are

YFP-positive was stable for number and location of cells across individuals in slices placed in vitro at E14. Transgenic Thy-1/YFP mice were used to determine the effect of GABA receptor antagonists on the movement characteristics of fluorescently labeled VMN and ARC neurons. YFP expression driven by the Thy-1 promoter was best seen in the more sub-ventricular and medial populations of VMN and ARC neurons in the 250μm thick slices of the current study (Fig. 1A; higher magnification Fig. 1B). Numbers of YFP positive neurons seen in 20 slices from 16 litters averaged 23 +/- 2.4 neurons (VMN) and 19 +/- 4.6 neurons (ARC). The field of view for watching movement in 250μm thick slices did not include the entire VMN or ARC regions; therefore there is no total count for all fluorescently labeled cells in the regions of interest. There are also limitations in our ability to see YFP-positive neurons due to the thickness of the slices and the short exposure time (30ms) used to prevent photobleaching. Movement patterns of the neurons that could be followed were similar to patterns observed previously in a related mouse line (C57Bl/6J) with DiI labeled neurons (Dellovade et al., 2001); cells moved along dorsal/ventral and radial orientations throughout the developing VMN. Pharmacological manipulation of GABA receptors, either through administration of bicuculline ($GABA_A$ receptor antagonist), or saclofen ($GABA_B$ receptor antagonist), increased the rate of cell movement by approximately 20% ($F_{1,48} = 4.07$, $p < 0.05$; Fig. 1E). There were no differences seen between the two drug treatment groups or with the location of YFP cells in the nucleus. Sample tracings of individual cells illustrate the distance traveled based on the change in speed before (Fig. 1C), and after (Fig. 1D) drug treatment. For comparison, cells in the ARC region were also analyzed for average movement speeds before and after drug treatment. Moving neurons in this region did not respond to the addition of either bicuculline or saclofen (Fig. 1F). There was a significant difference in the baseline speed between neurons moving in the region of the ARC and VMN. YFP-positive cells in the ARC moved about 30% faster than those in the VMN

($F_{1,64}$= 5.45, $p < 0.02$). The addition of saclofen following bicuculline or bicuculline following saclofen to the slices did not have any additional effects on the rate of motion of cells in the VMN or ARC (data not shown). To control for non-specificity, a cannabinoid receptor -1 agonist (WIN-55, 100μM; Sigma-Aldrich) was also administered to live slices. The CB-1 receptor mRNA is highly expressed in the VMN of the developing rat (Berrendero et al., 1998a). There were no changes in the movement direction or speed of neurons exposed to this drug (data not shown).

To determine if the movement of YFP neurons was affected by the direct response of GABA on these neurons, we looked for the presence of GABA receptors on YFP-positive neurons. YFP-positive neurons in E15 sections were surrounded by a network of immunoreactive $GABA_B$ (Fig. 2C) and $GABA_A$ (Fig. 2F and I) receptor subunits in the VMN. GABA receptors are expressed predominantly in non-nuclear cellular compartments while YFP primarily is a cytoplasmic marker (YFP expression is also seen in the nuclear regions of neurons likely due to leakage of the fluorescent protein). White arrows indicate examples where distinct YFP neurons were surrounded by GABA receptor expression.

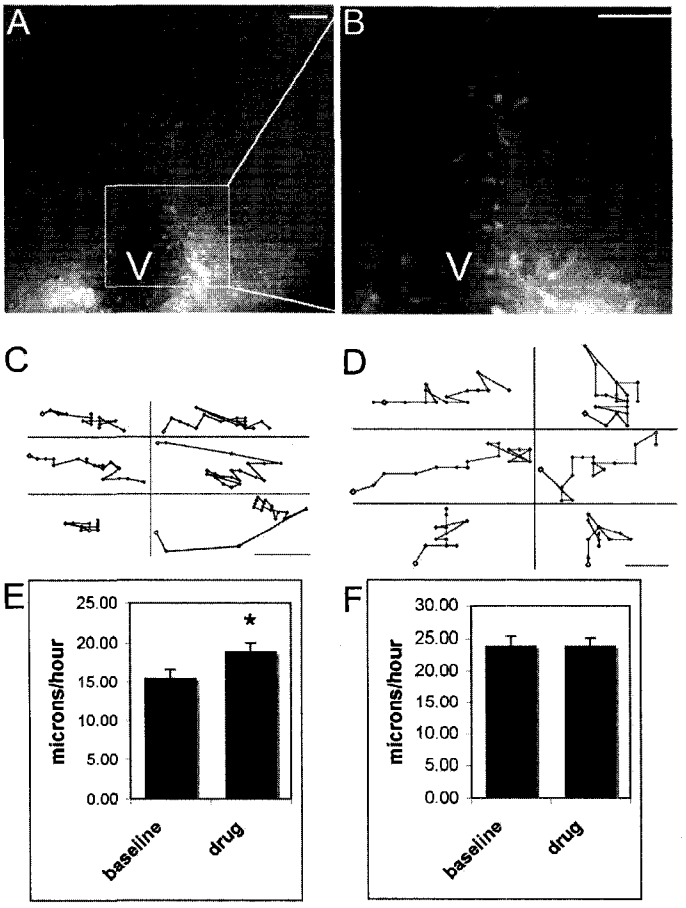

Figure 1 – Brain slices 250μm thick were used for the collection of video microscopy data indicating an increase in neuronal movement in the region of the developing VMN but not the ARC in response to GABA antagonists. The entire region of the hypothalamus that includes the VMN and ARC (A) has fluorescently labeled cells located near the ventricles (V). The boxed region represents the region magnified in (B; scale bars = 100μm). Migrating neurons did not follow strictly radial or dorsal/ ventral movement patterns, rather they changed directions and speeds numerous times within any given time period. Scatter plots for VMN (C) and ARC (D) cells represent sample neurons tracked over 90 minute time periods (scale bars = 5μm) with baseline shown on the left and drug response on the right of each panel. Movement did not occur between every frame and some cells appear to have moved more or farther than others. In the VMN (C,E) the baseline speeds were significantly slower than those observed following drug treatment (10μM bicuculline or 10μM-100μM saclofen; data combined and labeled as drug due to both drugs eliciting the same response). There was no change in the average rate of motion for neurons in the region of the ARC following GABAergic manipulation (F). The scales are different between panels E and F and ARC neurons moved notably faster than those in the region of the VMN.

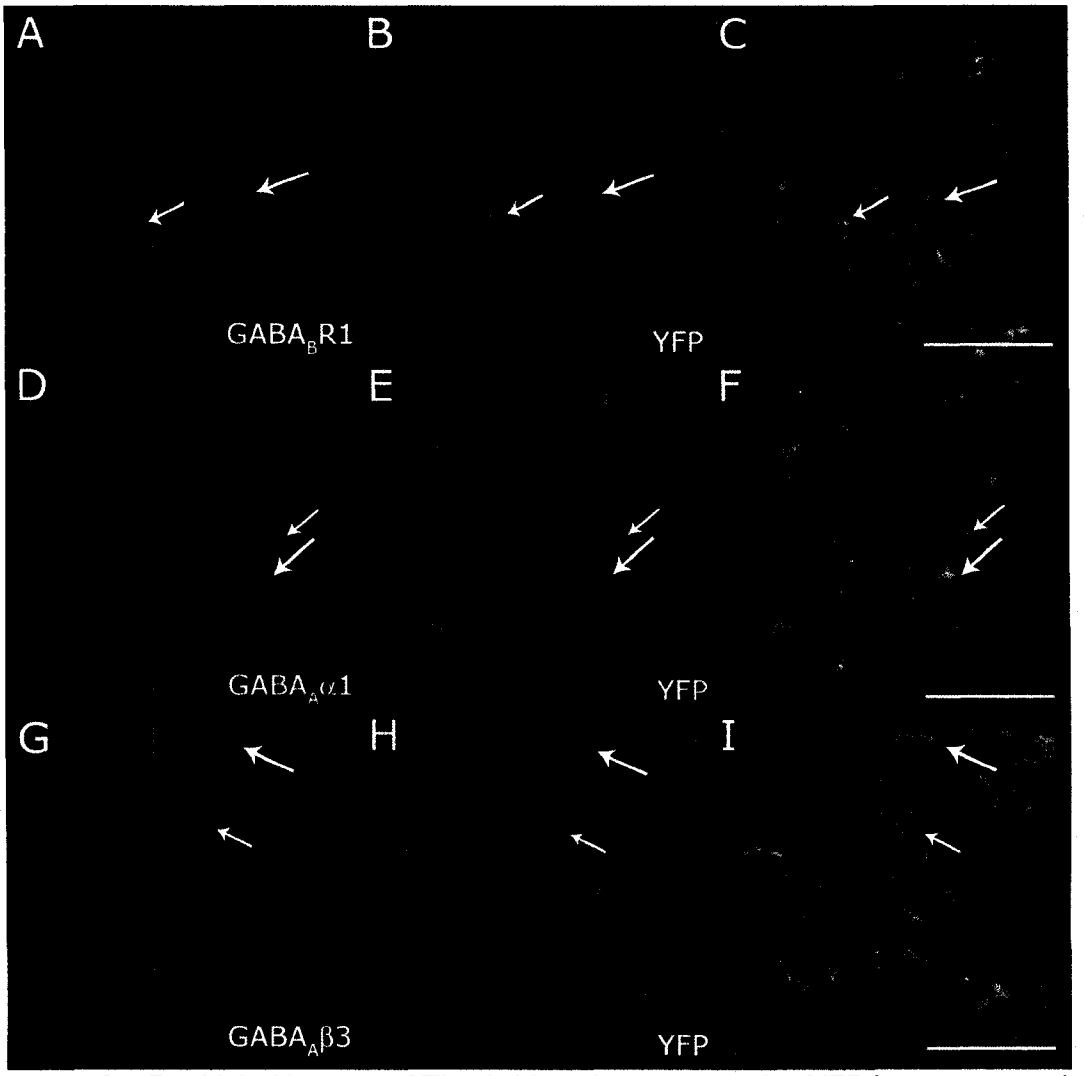

Figure 2 – Dual label images of Thy-1 driven immunoreactive YFP in neurons and GABA receptor subunits. Immunoreactive $GABA_B$ receptor subunit R1 (A), and $GABA_A$ receptor subunits α1 (D) and β3 (G) were found in both VMN and ARC (not shown) at E15. YFP containing neurons were surrounded by networks of GABA receptor subunit expression (C, F, I). White arrows indicate example cells that appear to show colocalization. For $GABA_B$R1 and $GABA_A\beta$3 subunits, immunoreactivity within the VMH was apparent in virtually all cells in non-nuclear compartment(s) whereas immunoreactive $GABA_A\alpha$1 subunits were found in a subset of cells (also in non-nuclear cellular compartments). Images were taken on a confocal microscope with a 1.6µm optical slice (scale bars = 50µm).

The distribution of lateral ERα immunoreactive neurons was changed in the $GABA_BR1$ knockout while more centrally located cell types (containing immunoreactive SF-1 or BDNF mRNA) were unchanged.

To test the hypothesis that alterations in GABA signaling in development affect final cell positioning, we examined brain sections from wild-type and $GABA_B$ receptor knockout mice for changes in immunochemically defined cell populations. We looked at the distribution of cells containing immunoreactive ERα throughout the VMN. They were densely located in the most ventrolateral region of the VMN at three different developmental ages - E17, P0, and P4. ERα immunoreactivity in the $GABA_BR1$ knockout at P0 appeared to shift dorsal to the expression pattern seen in the wild-type (Fig. 3C and D; genotype x location, $F_{(10,90)}= 3.61$, $p < 0.01$). Although cells were located in more dorsal positions, there was no change in the amount of total immunoreactive area [WT- 30,718 +/- 1568 mean pixels^2, KO- 23,954 +/- 1271 mean pixels^2; ($p > 0.10$)]; the mean total area (mean pixels^2) of ERα expression remained the same. The change in position that became evident at P0 remained at P4; we saw the same effect, with a more dorsal shift of ERα positive cells in the knockout as compared to the wild-type (Fig. 3E and F) with no difference in the total immunoreactive area [WT- 29,785 +/- 3765 mean pixels^2, KO- 25,437 +/- 2974 mean pixels^2]. At E17, we saw a pattern of immunoreactivity that looked similar to that seen at P0 and P4, however, the difference between genotypes was not statistically significant at this age (Fig. 3A and B) and the total immunoreactive area was unchanged [WT- 27,091 +/- 3836 mean pixels^2, KO- 25,559 +/- 5299 mean pixels^2]. Figure 4 shows a graphical representation of the dorsal shift seen in immunoreactive ERα. There is no significant difference in any of the rows at E17 (Fig. 4A). At P0, the largest significant difference in ERα immunoreactivity is between 100 and 200μm from the base of the brain (Fig. 4B). A similar pattern is seen at P4 (Fig. 4C), with

the largest significant difference between 200μm and 300μm from the base of the brain. There were no positional changes (medial/lateral or dorsal/ventral) based on ERα immunoreactive cells in the ARC at any age examined. We also measured the mean total area of ERα in the region of the ARC and found no difference between wild type and $GABA_BR1$ knockout animals at any of the three ages [E17; WT- 30,101 +/- 894 mean pixels^2, KO- 28,444 +/- 1420 mean pixels^2, P0; WT- 30,170 +/- 3299 mean pixels^2, KO- 32,570, +/- 5612 mean pixels^2, P4; WT- 38,020 +/- 3611 mean pixels^2, KO- 41,267 +/- 3660 mean pixels^2 ($p > 0.10$)].

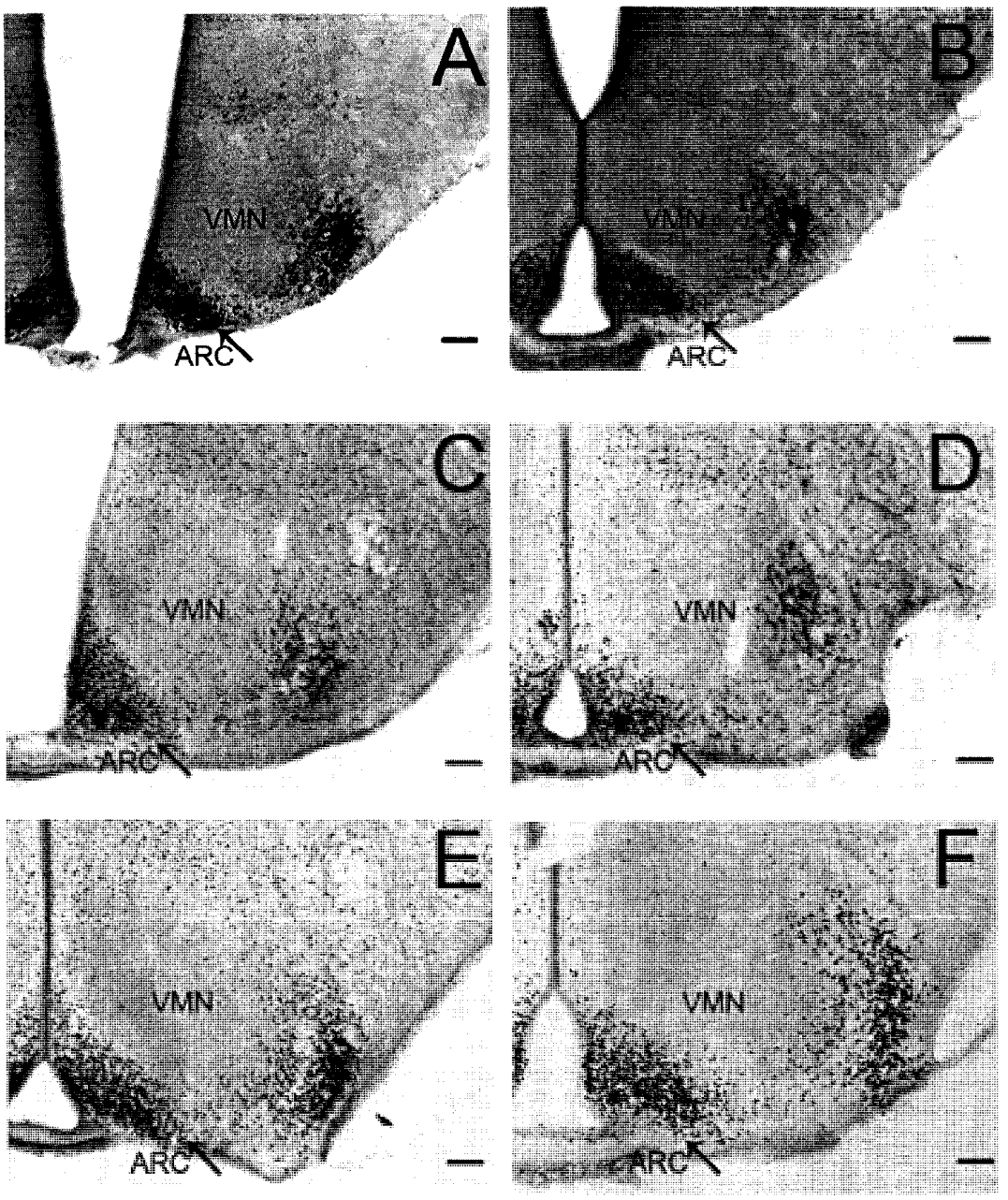

Figure 3 – Images show immunoreactive ERα in sections through the center of the VMN examined at 3 different developmental ages (E17, P0, and P4) in wild-type (A, C, E) and $GABA_BR1$ knockout (B, D, F) mice revealed a significant difference in the distribution of ERα positive cells in the ventrolateral quadrant of the VMN. At E17 there was no difference in the position of ERα immunoreactive cells (A and B). At P0 (C and D) and P4 (E and F) there was a more dorsal shift of cells containing immunoreactive ERα in the knockout animal. There was no significant change in ERα expression in the ARC.

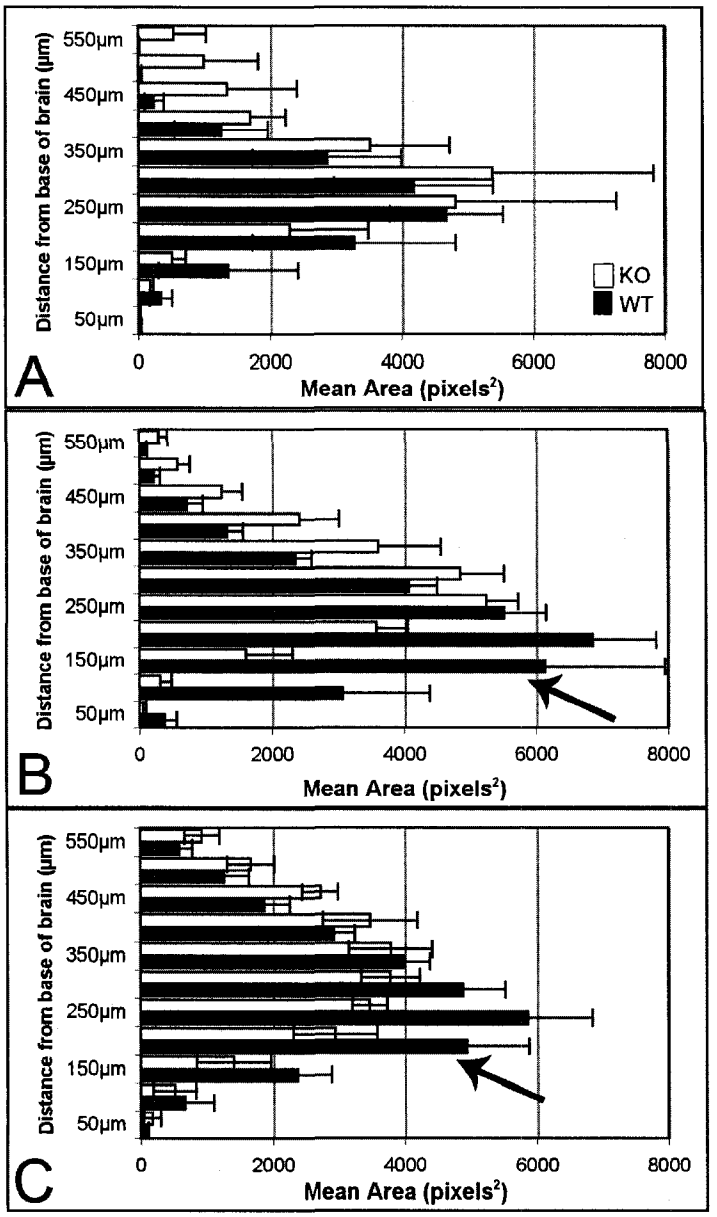

Figure 4 – Graphic representation of the area of ERα immunoreactivity in $GABA_BR1$ wild-type and knockout mice at 3 developmental ages. Bar graphs illustrate the immunoreactivity seen in each row based on a grid system with the first row along the base of the brain (n=6 for each genotype at each age). At P0 (B), the peak of immunoreactivity in the wild-type animal is between 150 and 200µm from the base of the brain and the peak of immunoreactivity in the KO animal is between 250 and 300µm from the base of the brain (scale bars = 100µm). At P4 (C), the peak of immunoreactivity in the wild-type is around 250µm from the base of the brain while the peak in immunoreactivity for the knockout animal ranged from 300-400µm from the base of the brain. There were significant differences between the wild-type and knockout animals at these two ages (genotype x position interaction, $p<0.05$). There were no significant differences at E17 (A).

To determine if GABA signaling through the $GABA_B$ receptor affects all cell types within the VMN, we also examined changes in the position (using the grid system as described above) or expression (total immunoreactive area) of neurons expressing immunoreactive SF-1 protein or BDNF mRNA (by in situ hybridization). SF-1 and BDNF are localized in

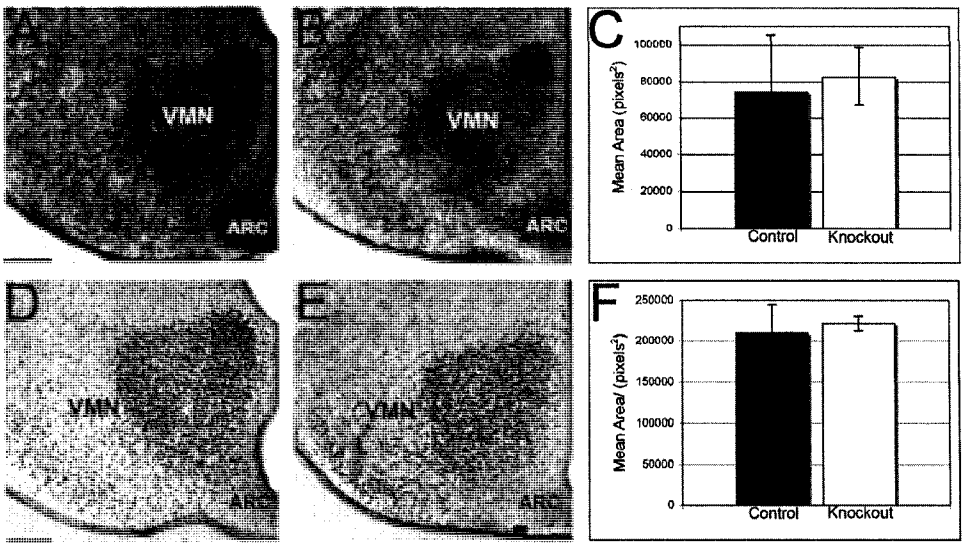

Figure 5 - BDNF and SF-1 expression in the VMN did not change in the $GABA_BR1$ knockout mouse at P0 (scale bars = 100μm). Wild-type (A, D) and $GABA_BR1$ knockout (B, E) mice were examined for changes in expression of BDNF mRNA (A-C) and immunoreactive SF-1 protein (D-F) located in the central and dorsomedial regions of the VMN. There were no apparent changes in total expression levels or the distribution of cells containing either marker.

cells of the more dorsal and central regions of the VMN (Tran et al., 2006). In the $GABA_BR1$ knockout the expression of these two markers was unchanged in both amount and position indicating that not all phenotypically identified cells of the VMN have altered positions in the $GABA_BR1$ knockout (Fig. 5).

To determine if the change in position seen in ERα positive cells in the ventrolateral quadrant of the VMN (vlVMN) of $GABA_BR1$ knockout mice was due to a disruption in radial fibers, sections containing regions of the VMN and ARC were processed for Thy-1 YFP (green) immunoreactivity and RC2 immunoreactive radial fibers (red). Tissue

sections were taken from a mouse line generated by crossing the $GABA_BR1$ heterozygote with a Thy-1/YFP mouse resulting in mice that were knockouts, heterozygotes, or wild-type for the $GABA_BR1$ gene and contained YFP expressing Thy-1 neurons. There were no apparent alterations of radial fibers in the $GABA_BR1$ knockout mouse (Fig. 6B) as compared to those in wild-type littermates (Fig. 6A). The presence of intact radial fibers suggests that the altered positions of ERα immunoreactive neurons in $GABA_BR1$ knockout mice was not due to the lack of or the severing of fibers that guide neuronal migration.

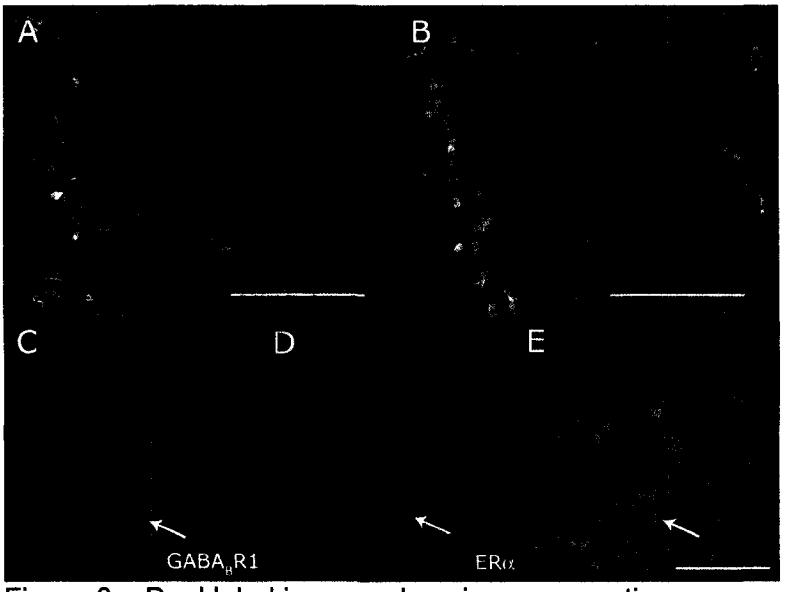

Figure 6 – Dual label images show immunoreactive Thy1/YFP (green) and RC2 (red) in the $GABA_BR1$ wild-type (A) and knockout (B) mouse. Radial fibers were intact in the knockout mouse suggesting that altered neuronal movement seen in Thy1/YFP-positive neurons was not due to severed or misdirected radial fibers (scale bars = 100μm; standard epifluorescence microscopy). $GABA_BR1$ (C) and ERα (D) appear to localize in the same cells (example at white arrow). As $GABA_BR1$ is a receptor found on the cell surface and ERα is a nuclear receptor it is difficult to conclude definitive colocalization. Images C, D, and E were taken using a confocal microscope with an optical slice of 1.6μm.

A small subset of Thy-1 YFP-positive neurons express islet-1 and ERα in the VMN and ARC

To determine if neurons containing YFP also expressed ERα, we utilized tissue sections from E15 Thy-1/YFP mice to look for colocalization. In the VMN (Fig. 7C) we found 3.3% +/-1.2 (n = 4) of YFP neurons colocalized with immunoreactive nuclear ERα. In the ARC (Fig. 7F), there was more colocalization of ERα and YFP (6.7% +/- 2.4, n = 3), which may be

due to the abundance of YFP neurons in the ARC at this age. ERα and islet-1 immunoreactive neurons localize to the ventrolateral quadrant of the VMN where most islet-1 neurons are also ERα immunoreactive (Davis et al., 2004b). There was no significant difference in the amount of YFP-positive neurons that colocalized with islet-1

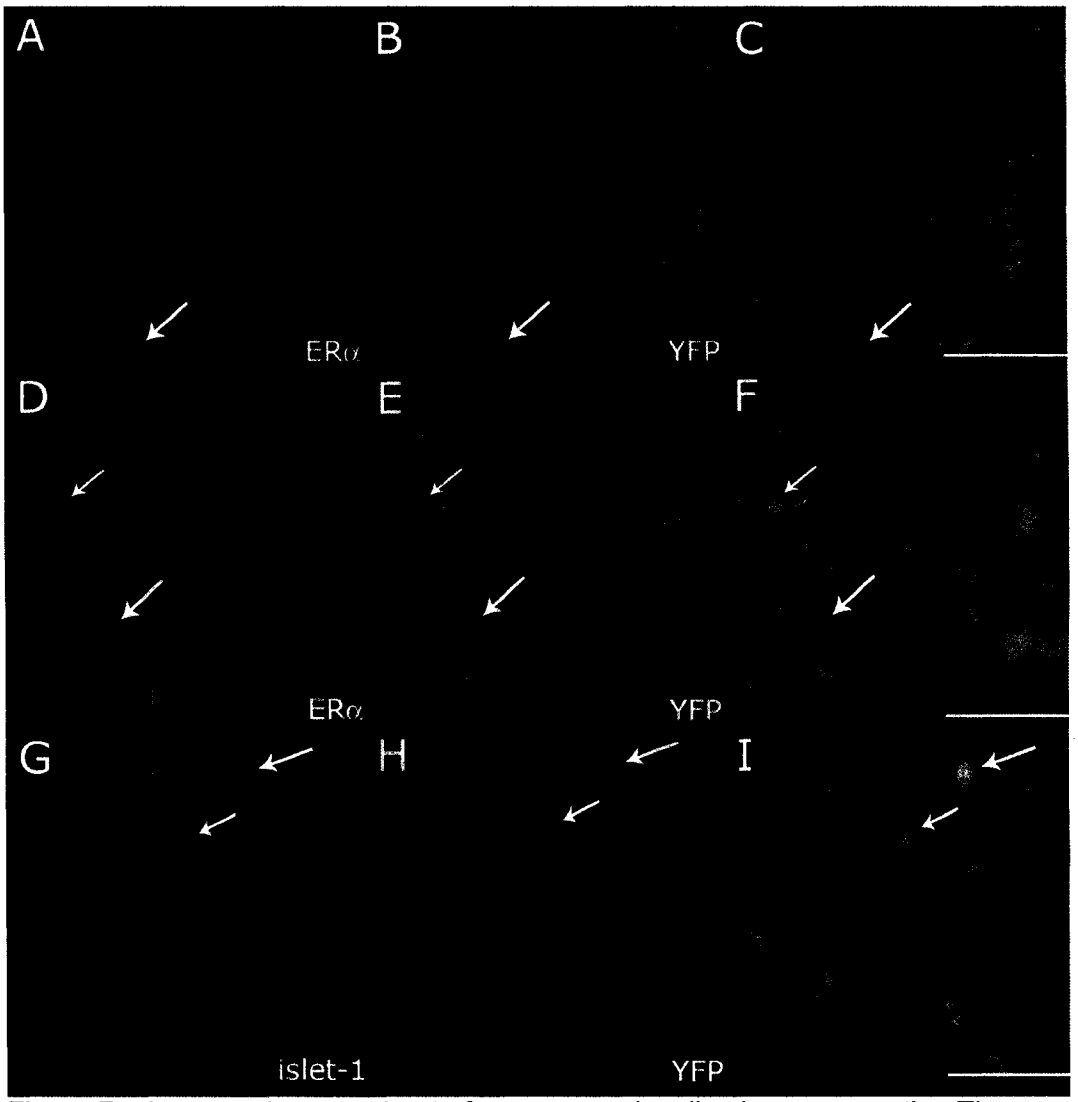

Figure 7 – Images show a subset of neurons co-localize immunoreactive Thy-1/YFP with islet-1 or ERα. The ventrolateral VMN contains a small population of ERα immunoreactive neurons at E15 (A) while the ARC contains a larger population of ERα cells (D). For the most part these cells co-localize (white arrows) in only a small number Thy/YFP neurons in the VMN (C) and ARC (F). Islet-1 is also expressed at developmental ages in the ventrolateral VMN and ARC. At E17 there was a subset of cells containing immunoreactive islet-1 (G) that were also Thy-1/YFP-positive (H, I). White arrows indicate examples of cells that colabel with YFP (scale bars = 50μm).

versus ERα in the VMN (7.9% +/- 2.9) or the ARC (13.9% +/- 7.5) (white arrows in Fig. 7I). Despite the low levels of YFP-ERα and YFP-islet-1 colocalization at E15, it is still possible that a number of the YFP neurons viewed in live video microscopy become estrogen receptor positive at later points in development. However, at the time points examined, they are not estrogen receptor positive and may represent other cell phenotypes within the VMN and ARC.

Discussion

The diencephalon can be parceled into major regions based on transcription factor expression (Lim and Golden, 2007; Puelles and Rubenstein, 2003) and some information is emerging relative to expression of transcription factors within the hypothalamus (Caqueret et al., 2006). Much less, however, is known concerning the details by which specific nuclear groups emerge from early transcriptional coding (McClellan et al., 2006; Xu and Fan, 2007). The VMN presents a unique opportunity to study the formation of a group of cells with a defined involvement in various aspects of neuroendocrine physiology and behavior. Previous studies have described the expression of the transcription factor SF-1 restricted within cells of the VMN while GABAergic elements surround the developing nucleus, providing a potential source of spatial patterning (reviewed McClellan et al., 2006). The results of the current study emphasize GABA's role in VMN development in contrast to the neighboring ARC and the role of $GABA_B$ receptors in VMN development. Figure 8 presents a model illustrating VMN development with and without alterations in $GABA_A$, $GABA_B$, or SF-1 function. To simplify this complicated system, the model focuses on the positioning of cells containing immunoreactive ERα and the movement characteristics of migrating neurons. As illustrated (Fig. 8B), loss of SF-1 causes the largest shift in cell locations with cells

containing immunoreactive ERα becoming located more medial (Dellovade et al., 2000; Majdic et al., 2002), in concert with a more medial pattern of immunoreactive glutamic acid decarboxylase (GAD) and GABA (Dellovade et al., 2000). By contrast loss of sensitivity to GABA, either by losing $GABA_A$ receptor function (Dellovade et al., 2001) or $GABA_B$ receptor function (current study) results in changes at the lateral edges of the VMN (Fig. 8C, D). The actions of GABA cannot explain all of VMN formation (e.g., no influence of GABA receptor modifications on medial and centrally located cells containing immunoreactive SF-1; Fig. 5 and Fig. 8). As an effective signaling molecule, however, it likely plays a key role in

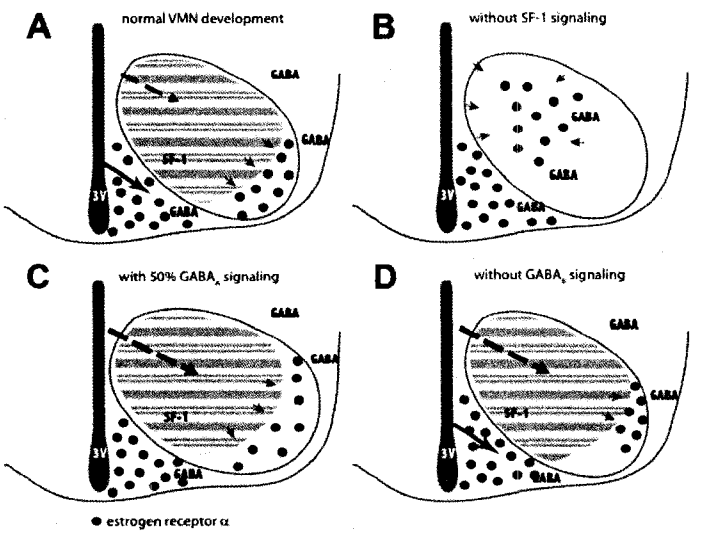

Figure 8 - In "normal" VMN development (A) cells migrate laterally, away from the third ventricle. Based upon the cell phenotype, VMN neurons occupy different subdivisions of the nucleus. As illustrated, SF-1 containing neurons occupy the dorsal and central regions of the nucleus while ERα containing neurons occupy the ventrolateral quadrant. Most cell movement throughout the nucleus is radial but there is some dorsal ventral movement, therefore ERα neurons may move in both directions before ending up in their final positions. Dotted arrows indicate movement speed of neurons migrating away from the third ventricle. Based on video microscopy data we know that VMN neurons moving away from the third ventricle move at an average rate of 15μm/hour. When SF-1 signaling is absent there is a medial shift in the positioning of ERα immunoreactive cells coincident with a medial shift of GABAergic neurons and fibers (Dellovade et al., 2000). Panels C and D illustrate changes when GABA signaling is inhibited. When $GABA_A$ signaling is reduced (C) there is an increase in the overall size of the VMN (Dellovade et al., 2001), ERα immunoreactivity in the ventrolateral quadrant is spread out and shifted slightly more dorsal. Based on data in the current study a lack of $GABA_B$ receptor signaling (D) does not change SF-1 expression throughout the dorsal and central VMN but does result in changes in the ventrolateral ERα population.

fine-tuning the positions of some VMN neurons and ultimately their connectivity.

The results of the current study provide evidence that endogenous GABA signaling affects the rates of cell motion in the region of the developing VMN, but does not alter the rate of motion in the ARC. GABA receptor antagonist administration led to an increase in the rate of motion in cells of the VMN suggesting that normal GABA signaling decreases or inhibits cell motion. These results were seen in fluorescently labeled Thy-1/YFP cells, located near the ventricular zone, which represent a subpopulation of VMN neurons. At E14/15, the ages used for live video microscopy (Figure 1) and dual label immunocytochemistry (Figure 2), the VMN and ARC are becoming distinct nuclear regions through the movement, positioning, and differentiation of various cell types. There is little evidence that these cells within the VMN, or those that will project to the VMN have begun to extend processes. Evidence of VMN projections had been described at 1 week postnatal with processes projecting from the amygdala to the VMN (Choi et al., 2005). Early signs of dendritic development in the rat VMN were also seen as primarily postnatal (Crandall et al, 1989). An alteration in migratory responses could be related to alterations in connectivity as suggested in discussions of roles for *Reelin* in hippocampal development (Del Rio et al., 1997). Determining influences on connectivity is a topic that needs to be addressed based on the current results.

The mechanism(s) through which GABA may act as a migratory cue involves direct ligand/ receptor interactions that ultimately influence cells to change rates of motion. The administration of GABA receptor antagonists increased the average speeds of neurons that were already moving in the slice preparation (motion when all frames averaged), but did not alter the likelihood of cell movement (percent of frames with motion). GABA can act through two different receptor subtypes that utilize two different mechanisms, but in

the current results, they resulted in the same effect on cell motions. $GABA_B$ receptors are metabotropic G-protein coupled receptors (Gi/o) that activate cGMP-signaling pathways, activating potassium channels and altering calcium currents, changing the calcium concentration within cells (Bowery and Brown, 1997). These receptors are different in both structure and mechanism from $GABA_A$ receptors, which signal through the movement of chloride along its concentration gradient, changing the membrane potential of cells. However, since chloride moves outward in embryonic neurons causing depolarization and potential calcium entry (Gao and van den Pol, 2000), one possible common mechanism through which GABA may be influencing cell speed is through changes in intracellular calcium levels. The location of $GABA_A$ and $GABA_B$ receptors on the same cells in hypothalamic neurons has been shown to influence the behavior of the neurons. $GABA_B$ receptor mediated inhibition has been shown to affect $GABA_A$ receptor mediated calcium spikes in embryonic hypothalamic neurons (Obrietan and van den Pol, 1998). In the current study, there was no additive effect of sequential addition of $GABA_A$ and $GABA_B$ antagonists further suggesting that the influence on motion was through a common mechanism.

The finding that cell motions are influenced by GABA signaling leads to the hypothesis that GABA signaling plays a role in determining cell positions within the VMN. GABA receptor antagonists influenced movements independent of the location within the developing nucleus. Thy-1/YFP labeled neurons examined in our live video microscopy studies move in two distinct directions; dorsal/ventral parallel to the third ventricle, and laterally along radial glial fibers. The majority of the fluorescently labeled cells examined in this movement analysis were located close to the third ventricle. When the data was analyzed separately for location (along the third ventricle or slightly more lateral) there were no differences in movement speeds between locations or in the orientation of the

cells (data not shown). As the majority of VMN neurons express specific subunits of the $GABA_A$ receptor and/ or the $GABA_B$ receptor (Davis et al., 2002; Dellovade et al., 2001), and the response to drug administration was relatively rapid, it is likely that the drug influence was direct to the moving cells. The specific subpopulation of YFP neurons being tracked for motion appears to be neurons that contain GABA receptors themselves. $GABA_A$ and $GABA_B$ subunit immunoreactivity appears to be in YFP containing neurons. It is difficult to determine if these are the same cells due to the pattern of immunoreactivity with GABA receptors apparent in non-nuclear cellular compartments and YFP being largely cytoplasmic. Cell movement in the ARC was measured before and after GABA receptor manipulation. Although the ARC also contains neurons expressing GABA receptors (Dellovade et al., 2001), cells in this region did not respond to GABA antagonists. This may be caused by a desensitization to GABA as many neurons in the ARC either synthesize GABA themselves or are surrounded by fibers and neurons that synthesize GABA (Gonzalez-Maeso et al., 2003).

The results of the present study expand on the role $GABA_B$ receptors play in the development of the VMN (Davis et al., 2002) by defining changes in VMN cell positions in mice with disruption of the $GABA_B$ R1 subunit. The results show significant changes in the positions of cells containing immunoreactive ERα in the ventrolateral VMN in the absence of functional $GABA_B$ receptors (Figs. 3, 4, 8D). The R1 subunit of the $GABA_B$ receptor contains the ligand binding domain for the $GABA_B$ receptor, therefore, the disruption of this subunit globally effects the function of this receptor and its ability to bind GABA (Jones et al., 1998). The pharmacological effects of $GABA_B$ antagonism (increased motion; Fig. 1) on the movement of Thy1/YFP cells in vitro is a logical candidate as a mechanism for cell displacement in the $GABA_B$ R1 subunit knockout mice. Although immunoreactive ERα and islet-1 was seen in few YFP containing

neurons at E15, Thy-1/YFP neurons were found in the ventrolateral quadrant of the VMN. The matching of YFP fluorescence to identified lateral VMN components was incomplete at the ages that motion was examined due to either low expression or antisera ability to detect immunoreactive ERα. The islet-1 immunoreactive cell population at E15 represented a greater population of ventrolateral neurons and therefore more colocalization with YFP may have been due to an increase in the number of islet-1 immunoreactive cells in the ventrolateral VMN. Nonetheless, it is likely that neurons that express ERα are influenced by the surrounding gradient of GABA based on several lines of evidence. First, a previous study showed a more widespread localization of cells containing immunoreactive ERα in mice lacking the β3 subunit of the $GABA_A$ receptor (Dellovade et al., 2001; Fig. 8C). Genetic disruption of this subunit has been shown to decrease $GABA_A$ signaling by up to 50% (Homanics et al., 1997). Second, in SF-1 knockout mice there is a massive reorganization of the region of the VMN and one of the first things altered is the pattern of GABAergic elements (Dellovade et al., 2000; Fig. 8B). Finally, in the current study, genetic disruption of the R1 subunit of the $GABA_B$ receptor resulted in a dorsal shift in cells containing immunoreactive ERα (Fig. 8D). More studies are needed to determine other subgroups of cells that co-label with YFP and that may be influenced by secreted signaling molecules like GABA. Interestingly, cells containing BDNF mRNA or immunoreactive SF-1 that are localized to the dorsomedial and central regions of the VMN were not influenced by the absence of functional $GABA_B$ receptors.

The response to antagonist in the current study is further evidence that cells in the region of the VMN respond to endogenous GABAergic signals. Previous work showed that immunoreactive GAD continues to encircle the developing VMN in brain slices up to

3 days in vitro (Tobet et al., 1999). Transcription factors (e.g., SF-1) play key roles in determining pattern formation in the developing nervous system. However, transcription factors do not by themselves signal between cells in development; effector molecules like GABA may play key roles in translating nuclear decisions (e.g., glutamic acid decarboxylase transcription) into cellular behaviors (Edelmann et al., 2007). In the embryonic mouse brain, GABA is found in a number of regions, where it may play role(s) unlike its more widely studied function in adult brain. In development, GABA has been shown to be excitatory (Obrietan and van den Pol, 1995), as well as influencing processes such as neuronal proliferation and migration (Behar et al., 1998). The large amounts of GABA that are synthesized along with the lack of developed synapses lead to the belief that the role GABA plays as a paracrine factor during development of the hypothalamus is unlike its function at the synapse (Gao and van den Pol, 2000; Manent and Represa, 2007; Taylor et al., 1990). GABA receptors are expressed within neurons as early as E13 and are likely capable of responding to GABA that may be secreted at the boundaries of the VMN at these early ages.

In summary, the current study has taken two independent lines of evidence to converge on the potential role of GABAergic stimulation on hypothalamic nuclear development. In vitro evidence showed that the manipulation of GABA receptors altered the motion of a specific subpopulation of neurons within the VMN but not the ARC. This result provides evidence that GABAergic stimulation is relevant to specific regions in the hypothalamus (VMN) while other regions, for reasons yet to be determined, are not responsive. The same result (an increase in motion) was seen upon the addition of either $GABA_A$ or $GABA_B$ antagonists leading to the conclusion that although the initial mechanisms may differ, GABA action through either receptor may ultimately invoke a common mechanism. In vivo evidence using mice in which the $GABA_B$ receptor was disrupted

showed that mice lacking this receptor in development exhibited phenotypic changes within the hypothalamus; including, changes in the position of cells in the VMN suggesting significant long term consequences of these developmental actions. These lines of evidence emphasize the importance of GABA and GABA receptors in the development of specific regions or nuclei within the hypothalamus. Further experiments are needed to determine if altered movement eventually leads to altered functional characteristics or synaptic connections within the developing brain

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Chapter 4

Prenatal development of the paraventricular nucleus of the hypothalamus and potential roles for gamma-aminobutyric acid and Brain Derived Neurotrophic Factor

Abstract

The development of the hypothalamic paraventricular nucleus (PVN) involves several factors that work together to establish a cell group that regulates neuroendocrine functions and behaviors. In the current study immunoreactive GABA was found in cells and fibers surrounding the PVN early in development. A $GABA_BR1$ subunit knockout mouse was used to look at cell positions in the absence of normal $GABA_B$ signaling. Estrogen receptor (ER)α immunoreactive (ir) neurons were found within the dorsal PVN and lateral to the PVN as early as E15. In female but not male $GABA_BR1$ subunit knockout mice the position of ir-ERα shifted from medial to lateral compared to wildtype while the total number of ir-ERα containing cells was not changed. Although the size of the PVN and the number of cells increases throughout development, a number of molecular markers were noted in the developing PVN, including brain derived neurotrophic factor (BDNF). Immunoreactive BDNF was examined in $GABA_BR1$ subunit knockout mice to begin determining the interactions of secreted factors on PVN development. There was a significant decrease in ir-BDNF in the knockout mice in a region dependent manner within the PVN at E17. Therefore, changes in cell position subsequent to disruption of $GABA_B$ signaling may be due, in part, to diminished BDNF signaling. Based on the current study, the PVN can be added as another site where

GABA exerts morphogenetic actions in development and, in particular, through $GABA_B$ receptor signaling.

Introduction

The paraventricular nucleus of the hypothalamus (PVN) lies at the dorsal limit of the classical hypothalamus at the base of the diencephalon. It has been implicated in a broad array of homeostatic and behavioral functions ranging from neuroendocrine and cardiovascular control to affective, ingestive, and defensive behaviors (Herman et al., 2005; Swanson and Sawchenko, 1983). Numerous peptides, neurotransmitters (e.g., corticotropin releasing hormone (CRH), arginine vasopressin (AVP), oxytocin (OT)); (Armstrong et al., 1980; Ford-Holevinski et al., 1991; Swanson and Sawchenko, 1983), and other proteins including calbindin (Brager et al., 2000) and neuronal nitric oxide synthase (nNOS) (Bernstein et al., 1998) characterize the chemoarchitecture of the PVN. Steroid hormone receptors are also among the markers located within and around the PVN. Thus, populations of cells that contain immunoreactive estrogen receptors-α (ir-ERα), ERβ, glucocorticoid (GR) and androgen (AR) receptors characterize select regions inside and surrounding the PVN (Mitra et al., 2003; Simerly et al., 1990; Suzuki and Handa, 2004).

The development of the PVN has been studied using Nissl stains, neuronal birthdating, and the identification of cell phenotypes at early ages. Based on Nissl staining, the PVN is first visible as a cell group between embryonic day (E)15 and E17 in mice (Karim and Sloper, 1980; Shimada and Nakamura, 1973). A number of the different cell phenotypes that delineate regions within the PVN are expressed early in development. Estrogen receptors and glucocorticoid receptors are among those found at embryonic ages (Owen and Matthews, 2003; Tobe et al., 2005). Neuroactive peptides such as oxytocin and

vasopressin (Okamura et al., 1983), transcription factors, neurotrophic factors (e.g., BDNF; (Fujioka et al., 2003), and neurotransmitters also are expressed by PVN neurons early in development (Michaud et al., 1998b; Xu and Fan, 2007). These cells are derived from precursors along the third ventricle with distinct timelines; more lateral (magnocellular) cells are primarily born between E10.5 and E12.5 in the developing rat (Altman and Bayer, 1986; Markakis and Swanson, 1997), with medial (parvocellular) cells being born later.

Differentiation of the PVN requires the expression of particular transcription factors, including Sim1 (Michaud et al., 1998b), Arnt2 (Michaud et al., 2000), Otp (Acampora et al., 1999; Wang and Lufkin, 2000), Brn2 and Nkx2.2 (Caqueret et al., 2006). In the absence of Sim1, there was a decrease in OT and AVP expression in the PVN and the supraoptic nucleus (SON) suggesting that Sim1 is required for terminal cell differentiation of these cell types in both the PVN and SON (Michaud et al., 1998b). More recently it has been suggested that Sim1 expression may also influence cell migration within the region of the PVN (Xu and Fan, 2007). Cell proliferation as indicated by bromodeoxyuridine incorporation and cell death as indicated by TUNEL positive cells were similar in Sim1 mutants compared to wildtype mice, however, the distribution of Sim1 presumptive cells in the mutant indicate an altered migratory phenotype that may be mediated through the direct regulation of PlexinC1 (a receptor known to be involved in migration and axon guidance) by Sim1 (Xu and Fan, 2007).

For mechanisms of cell migration to drive the formation of the PVN, molecular communication between cells must come from small molecules or proteins downstream of transcription factors. Several neurotransmitters/neuropeptides including, GABA, serotonin, dopamine, and endogenous opiates have been suggested to act as

neurotrophic factors or morphogens in various brain regions (Lauder, 1993; Nguyen et al., 2001). One of the potential cues involved in migration within the hypothalamus is the neurotransmitter GABA (Tobet et al., 1999). GABA has been shown to influence migration within one hypothalamic nucleus, the ventromedial nucleus (VMN), through $GABA_A$ and $GABA_B$ (McClellan et al., 2008) receptors. The distribution pattern of GABAergic cells and fibers surrounding the region of the developing VMN provides potential boundary information for influencing cells moving into the ventrolateral region of the nucleus (McClellan et al., 2006). The current study examined the relationship of GABAergic cells and fibers surrounding the developing PVN to determine if similar roles might be in play.

To test the hypothesis that GABA plays a role in PVN development, the distribution of specific cell types was examined in the PVN of a $GABA_BR1$ receptor knockout mouse that we had previously analyzed for VMN development (McClellan et al., 2008). Immunoreactive ERα was found in cells of the dorsal and medial region of the PVN and also in a population of cells lateral to the PVN (lateral hypothalamus/ perifornical region). BDNF is found early in development within the PVN and is a key neurotrophic factor controlling diverse brain functions in development and in adulthood. BDNF is a member of the neurotrophin family of proteins with activity mediated by binding to a specific receptor tyrosine kinase (TrkB) as well as the non-selective receptor $p75^{LNTR}$ (Tapia-Arancibia et al., 2004). BDNF has been shown to be an important molecule in the development and differentiation of the central and peripheral nervous systems, including having an influence on neuronal migration (Borghesani et al., 2002; Zhou et al., 2007). The PVN contains high levels of BDNF and TrkB mRNA and these overlap substantially with the distribution of AVP and CRH (Givalois et al., 2004). In prenatal hypothalamic neurons, BDNF influences the release of GABA, while in turn, GABA release stimulates

BDNF expression (Obrietan et al., 2002). In the current study, we show that GABAergic elements surround the developing PVN and that $GABA_B$ receptors may play a role in determining the positions of cells containing immunoreactive ERα inside and outside the PVN in females. We also show that $GABA_BR1$ subunit knockouts exhibit a selective decrease in BDNF expression in the PVN leading to the hypothesis that GABA and BDNF may act synergistically to determine aspects of PVN development.

Materials and Methods

Animals

Mice used in this study were all from lines generated on a C57BL/6J background. The majority of the experiments used a transgenic line of mice lacking functional $GABA_B$ receptors through disruption of the gene for the R1 subunit (Prosser et al., 2001). Heterozygous breeding pairs were used to generate homozygous null, heterozygous, and wild type animals to be used for Nissl staining and immunohistochemistry. Animals were mated overnight and checked for vaginal plugs the following morning. The day of plug was designated as embryonic day (E)0. Pups were transcardially perfused on either day E15, E17 or postnatal day (P)0. Pregnant mice were anesthetized with a combination of ketamine (80mg/kg) and xylazine (8mg/kg) and embryonic pups were removed individually before being perfused with 2mL (E15 and E17) or 5mL (P0) 4% paraformaldehyde. Body weight and crown rump length measurements were taken to verify ages. Sex determination was made through direct inspection of the gonads or PCR analysis for the Y-chromosome *sry* gene. Brains were postfixed in 4% paraformaldehyde overnight and were placed in 0.1M phosphate buffer and stored at 4°C until tissue slicing.

Genotyping

Genotyping of tail DNA was done as described previously (McClellan et al., 2008). Mice were genotyped for the $GABA_BR1$ knockout allele and the *sry* gene using a standard Taq polymerase PCR kit (Qiagen, Valencia, CA).

Nissl staining and nucleoli analysis

Brain tissue collected from pups at ages E15, E17, and P0 were embedded in 5% agarose and cut into 50μm thick coronal sections using a vibrating microtome (VT1000S; Leica Microsystems, Wetzlar, Germany). Tissue sections were mounted onto glass slides pre-coated with gelatin and left to dry at room temperature overnight. Sections were rinsed in 50% ethanol, rehydrated in distilled water, and stained with a 0.1% thionin solution. Sections were rinsed in 70% ethanol with glacial acetic acid for color differentiation. Sections were then dehydrated in a graded series of ethanol washes, placed in xylene, and then coverslipped using Permount (Fisher Scientific, Waltham, MA). Sections were processed for immunohistochemistry and counterstained follow the same Nissl procedure described here but were first rehydrated in a graded series of ethanol washes and distilled water after soaking off coverslips in xylene. To image thionin stained nucleoli within individual cells, high magnification images were taken on an Olympus BH2 microscope with an Insight QE digital camera using Spot Advanced software (Spot Business Systems, Draper, UT). For each E15 Nissl stained group of sections, the PVN region was determined based on various anatomical landmarks including the appearance of the third ventricle at the point of flexure. A cell poor zone surrounds the PVN region and was used to designate the outer boundary. The number of cells with multiple nucleoli visible at 100x magnification was counted manually in 1 field (n=3). This was done for the medial PVN, lateral PVN, and region outside the PVN.

Immunohistochemistry

Brain tissue collected from pups at ages E15, E17, and P0 were embedded in 5% agarose and cut into 50μm thick coronal sections using a vibrating microtome (Leica VT1000S). Alternating sections were collected in 0.05M phosphate buffered saline (PBS). Excess unreacted fixative was neutralized using a 30-minute incubation in 0.1M glycine and 15 minutes in 0.5% sodium borohydride in PBS. After washing the tissue sections in PBS the sections were incubated in a PBS blocking solution for at least 30 minutes at 4°C containing 5% normal goat serum, 0.3% Triton-X 100 (Tx) and 1% hydrogen peroxide. Following the blocking step, the tissue was incubated in primary antisera containing 1%BSA and 0.3%Tx. Primary antibodies used were: rabbit polyclonal antisera directed against ERα at a dilution of 1:5000 (C1355; Upstate Biotechnology, Charlottesville, VA), rabbit polyclonal antisera directed against BDNF at a dilution of 1:200 (sc-546, N-20; Santa Cruz Biotechnology, Santa Cruz, CA), CRH (1:25,000; generously provided by Dr. Wylie Vale), vasopressin (1:10,000; Immunostar, Hudson, WI), ERβ (1:500; early lot number 10967002 of Z8P, Zymed Laboratories, San Francisco, CA), neuronal nitric oxide synthase (nNOS - 1:10,000, Immunostar), calbindin (AB1778,1:5000, Millipore, Billerica, MA and D-28K, 1:40,000, Sigma-Aldrich, St. Louis, MO), galanin (1:8000, Millipore (Brown et al., 1999), GABA (1:500, Immunostar), glutamic acid decarboxylase antisera (GAD; GABA synthesizing enzyme , GAD67, 1:5000, Millipore) and NPY (1:8000, Immunostar). Tissue sections were incubated over 2 nights at 4°C with primary antisera. Sections were washed at room temperature in PBS containing 1% normal goat serum and 0.02% Tx. Sections were incubated at room temperature in secondary antisera buffer containing 1% normal goat serum and 0.32% Tx with a biotin conjugated anti-rabbit secondary diluted to 1:2500 (Rabbit IgG-fab fragment; Jackson Immunoresearch, West Grove, PA). Sections were developed using a Vectastain ABC Elite kit (Vector Laboratories; Burlingame, CA) for 1 hour at room

temperature and visualized with a 5 minute incubation in a solution containing 0.025% diaminiobenzidine with 0.02% nickel and 0.02%H_2O_2 diluted in tris-buffered saline (pH 7.5). Pre-incubation with peptide served as an antibody control for BDNF (1µg peptide: 1µg antibody for 1 hour at room temperature, data not shown), while similarity of immunoreactivity among antisera served for others (e.g., calbindin, and comparison of GABA to GAD), similarity to literature reports for others (e.g., vasopressin, oxytocin, galanin), as well as omission of primary antisera that resulted in non-detectable reaction product.

Analysis

Images of the rostral PVN were taken at 10x magnification on an Olympus BH2 microscope with an Insight QE digital camera using Spot Advanced Software. Images were normalized for optimal contrast using Adobe Photoshop software (version CS for Macintosh). For the analysis of cells containing immunoreactive ERα and immunoreactive BDNF, images were opened in IP Lab Imaging software (Scanalytics Inc. part of BD Biosciences, Rockville, MD) and grids which include columns (100µm wide) and rows (100µm tall) were placed over the images with the boundaries being the edge of the third ventricle and the top of the PVN. For ERα analysis, areas were determined following standard segmentation of the most densely reacted cellular elements in each image. The area of immunoreactive cells was measured in each column. Totals were calculated for overall immunoreactivity and by column. For BDNF analysis, the images were normalized to ensure that the pixel intensities were spread across the dynamic range, and then the darkest one third of the pixels were segmented for quantification of the area occupied by positive cells (Davis et al., 2004). The area of immunoreactive cells was measured in each column. Totals were calculated only for boxes that were within the region of the PVN and those boxes that were outside of the

PVN were not included in the analysis. Column analysis was used to determine changes in location within the PVN. Statistical significance was determined by ANOVA for genotype x column (as a repeated measure) using SPSS software (SPSS Inc., Chicago, IL).

Results

PVN development at embryonic ages

The cellular organization of the mouse PVN emerges as a bilateral triangular shaped nucleus adjacent to the third ventricle (Fig. 1). The cytoarchitecture of the nucleus matures between E15, when it is visible as an increase in cell density along the wall of the third ventricle, and E17, when the PVN appears fully developed with distinct boundaries separating the nucleus from surrounding cell poor zones. Nissl stains at E15 (1A), E17 (1B), and P0 (1C) show the change in size and cell number through development. Higher magnification images at E15 show the presence of cells containing one or multiple nucleoli in the medial, lateral, and outer region of the PVN. Immature cells are likely to have multiple nucleoli versus mature cells that usually contain one (Helm et al., 1992). We found more

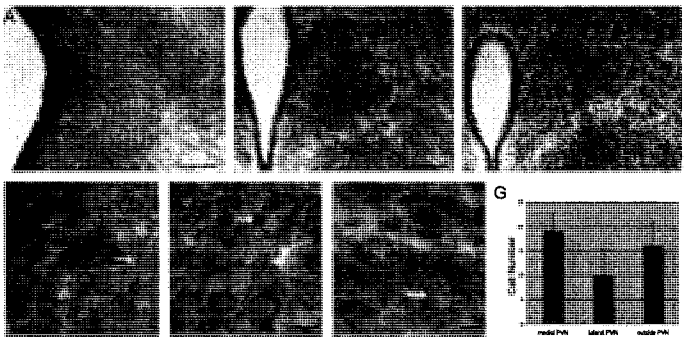

Figure 1 – Nissl stain sections of the region of the paraventricular nucleus (PVN) at embryonic day (E)15 (A), E17 (B), and postnatal day 0 (C) (scale bars =50μm). The PVN is visible as a density of cells adjacent to the ventricle at E15 (A) and progresses to be a well defined nuclear group surrounded by a cell poor zone (B, C). High magnification images of cells at E15 are shown within the medial (D), lateral (E), and outer (F) regions of the PVN (scale bars =10μm). Examples of cells with multiple nucleoli are marked with black arrows and those with single nucleoli are marked with white arrows. More cells within the medial PVN contained multiple nucleoli vs. those in the lateral PVN (G).

cells containing multiple nucleoli along the ventricular zone in the medial region of the PVN than in the lateral region of the PVN (Fig. 1D-G).

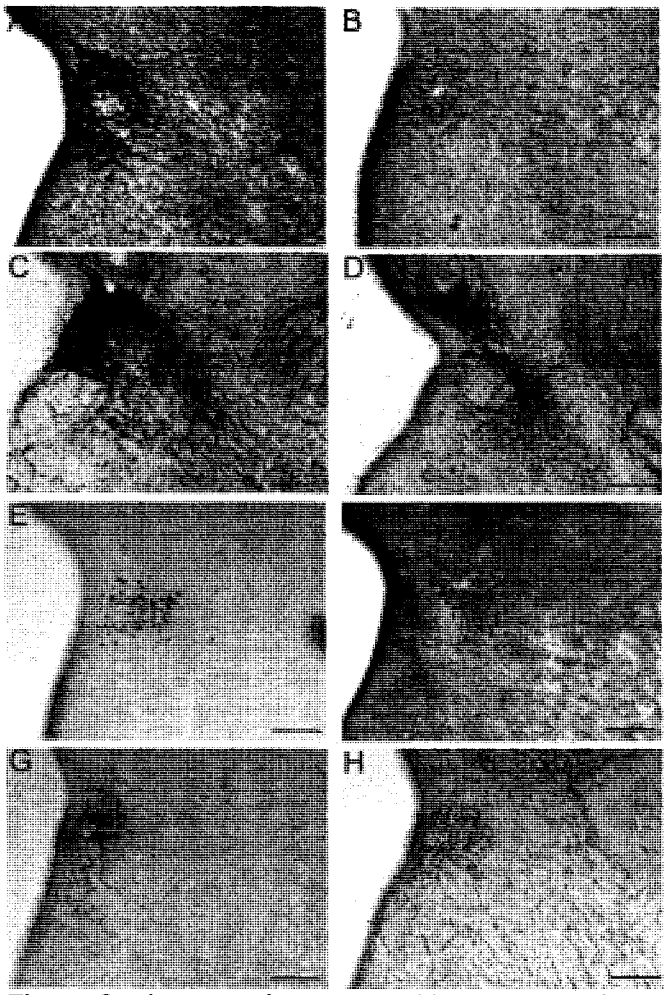

Figure 2 – Images show several immunoreactive peptides, receptors, and signaling molecules that populate the paraventricular nucleus (PVN) by embryonic day 15. Corticotropin releasing hormone (A), vasopressin (B), calbindin (C), neuronal nitric oxide synthase (D), estrogen receptor (ER)β (E), ERα (F), galanin (G), and neuropeptide Y (H) demarcated distinct populations of cells in the PVN at early ages (scale bars = 50μm). As the PVN developed, becoming larger in volume, the numbers of cells expressing these markers increased but with similar patterns of distribution.

PVN cell phenotypes at E15

While Nissl stain analysis revealed an immature nuclear group at E15 barely visible as an increase of cell density along the third ventricle, several immunochemical characteristics selectively delineate the developing PVN based on cytoarchitecture, prior to its emergence and lateral extension. Thus, many different cell phenotypes have

already established positions within the developing nucleus. Figure 2 shows the distribution of a subset of immunoreactive markers that have differentiated by E15 in the PVN. These include cells containing corticotropin-releasing hormone (Fig. 2A), oxytocin (2B), calbindin (2C), neuronal nitric oxide synthase (2D), ERα (2E), ERβ (2F), galanin (2G), and NPY (2H). Based on the distribution of these various cell types it is clear that the cellular architecture is established at young ages, however, the size of the nucleus and the number of cells occupying the PVN increases with further development (Fig. 5; ERα at E15, E17, and adult). Of the molecular markers examined, immunoreactive NPY was the only one not intrinsic to cells of the PVN. Unfortunately cell bodies of origin were not obvious. Of the remaining molecular markers for neurons intrinsic to the PVN, none had immunoreactive processes extending much beyond the nucleus through P0.

The relationship of GABA to the PVN

Cell bodies and fibers containing immunoreactive GABA or GAD67 surrounded the developing PVN as early as E13. This is apparently unique to two regions within the

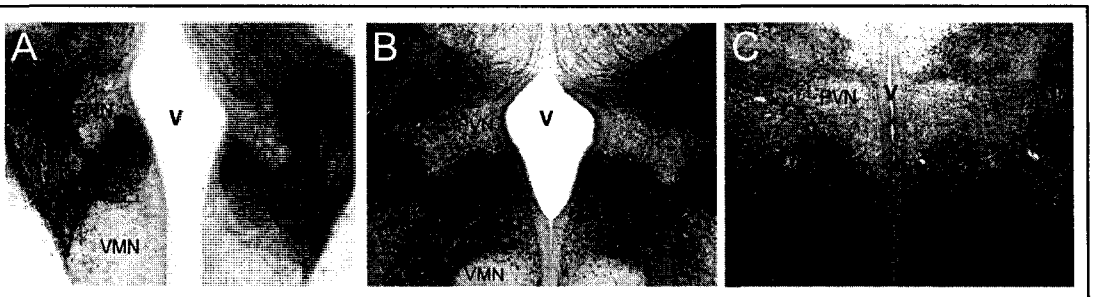

Figure 3 – Images show immunoreactive GAD 65/67 (A) or GABA (B-C) surrounded the region of the developing PVN at embryonic ages (scale bars = 100μm). At E13 (A), E15 (B), and E17 (C) in the mouse, the PVN region is one of two hypothalamic regions where GABA is found surrounding the nucleus in development. The VMN, the other nuclear group where this has been found, is shown within the images of panels A and B. By adulthood GABAergic fibers have moved into the region of the PVN and VMN (data not shown). PVN = paraventricular nucleus of the hypothalamus, VMN = ventromedial nucleus of the hypothalamus, V = third ventricle

developing hypothalamus - the PVN and the VMN. The progression from E13 (Fig. 3A),

to E15 (Fig. 3B), to E17 (Fig. 3C), shows the dense populations of GABAergic cells and fibers that surround the PVN. By adulthood, GABAergic fibers completely fill the nucleus (data not shown).

Female $GABA_BR1$ subunit knockout mice exhibit increased numbers of cells containing immunoreactive ERα lateral to the PVN.

Brains from mice lacking functional $GABA_B$ receptors were analyzed for ERα-ir at P0 in sections containing rostral and caudal sections of the PVN. A spatial analysis revealed a difference in the medial to lateral distribution of cells containing ir-ERα. To analyze this distribution of cells, 100μm wide columns were graphically placed over images of the tissue and pixel density was determined for each column. The columns were numbered 1-7 with column 1 being closest to the ventricle. $GABA_BR1$ knockouts were compared to wildtype animals and separated by sex. The data were analyzed as immunoreactive areas in a three-way ANOVA, sex by genotype by location considered as a repeated measure. The analysis showed a significant three-way interaction ($F_{(6,120)}=2.43$, $p < 0.05$) that clearly reflected the fact that a knockout phenotype was only evident in females, and was dependent upon location. In female knockout mice there was an approximate 80% decrease in ERα-ir within the fist 100μm from the ventricle ($p<0.05$) and an approximate 70% increase in immunoreactive area (proportional to cell number) between 500 and 600μm from the ventricle as compared to wildtypes ($p<0.05$; Fig. 4A-C). There were no differences in the distribution of ir-ERα in the same regions in males (Fig. 4D-F). Furthermore, there was no difference in the overall amount of ir-ERα, which included the PVN and the lateral population. Sections from brains of $GABA_A$ receptor β3 knockout mice (Dellovade et al., 2001) were re-examined for ir-ERα in and around the

region of the PVN. There was no significant difference in amount or location of ERα immunoreactive neurons in either sex (data not shown).

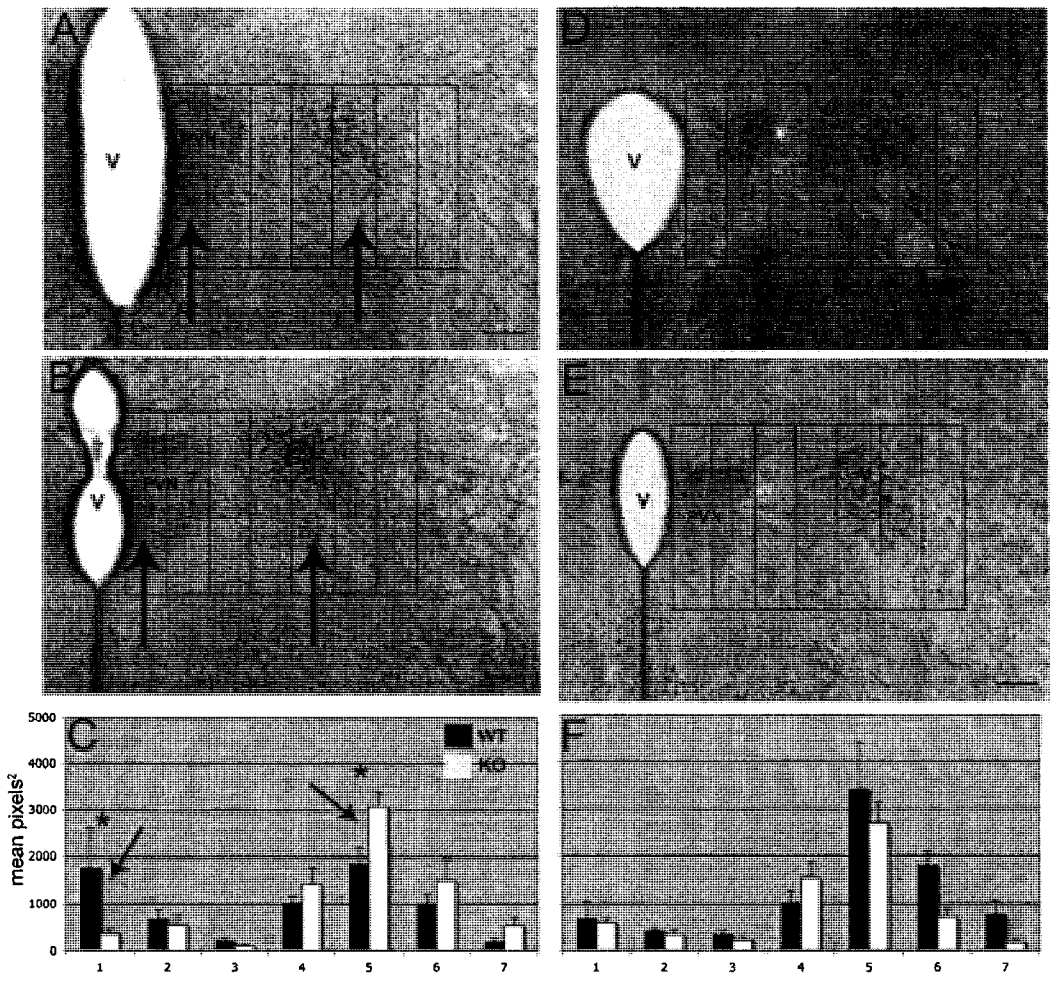

Figure 4 – ERα immunoreactivity at P0 in $GABA_BR1$ subunit wildtype (A,D) and knockout (B,E) mice. Seven 100μm wide columns were placed over images with the edge of the third ventricle and the top of the PVN as the boundaries for the grid. Female $GABA_BR1$ subunit knockouts (n=6) had an approximately 80% decrease in immunoreactivity in column 1, as measured by mean pixels^2, the first 100μm from the ventricle (A-C), and an approximately 70% increase in immunoreactive area in column 5 (400-500μm from the ventricle), versus wildtype littermates (n=6). There was no difference in levels of immunoreactivity in male $GABA_BR1$ subunit knockout mice (D-F; n=6) as compared to wildtype (n=6) (scale bars =100μm). V = third ventricle, PVN = paraventricular nucleus

Sections processed for immunoreactive ERα at E15 and E17 (Fig. 5A,B) were counterstained for Nissl substance to determine if the lateral cell group containing ir-ERα was inside or outside of the boundaries of the PVN. This cell group appears to be distant from the ventricle as early in development as E15 (Fig. 5A), more lateral still at E17 (Fig. 5B), and into adulthood (Fig. 5C). In adulthood, the columns of the fornix are clearly visible and this population of cells containing ir-ERα, outside the PVN, is located just dorsal to the descending columns of the fornix.

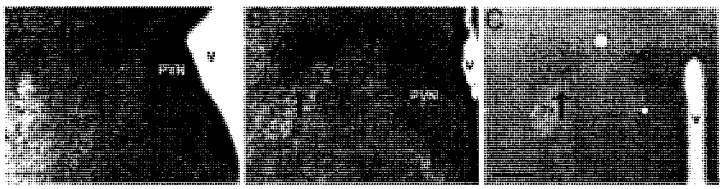

Figure 5 – Images of sections containing immuoreactive ERα, and that were then counterstained with thionin, show the boundaries of the PVN and the lateral group of ERα cells outside of these boundaries (E15 in A, E17 in B, scale bars =50μm). In the adult, this lateral group of ERα cells is still visible outside of the PVN and appears to be just dorsal to the columns of the fornix (C, scale bars = 250μm). V = third ventricle, Fx = columns of the fornix, PVN = paraventricular nucleus

Adjacent sections were processed for ir-ERα and ir-$GABA_BR1$ subunit to determine if those cells containing ir-ERα might have $GABA_B$ receptors. Although the cellular resolution is hampered by the distribution of a membrane protein, the R1 subunit of the $GABA_B$ receptor was found in cells within the boundaries of the PVN and was found laterally in the region where the ERα cells are positioned just outside the PVN (Fig. 6A, B).

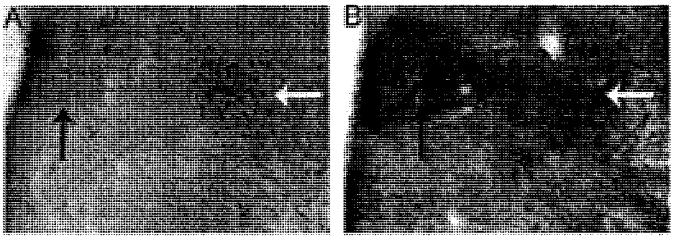

Figure 6 – Adjacent sections with ERα (A) and $GABA_BR1$ subunit (B) immunoreactivity at E17. Black arrows point to immunoreactivity within the region of the PVN and white arrows point to immunoreactivity lateral to the classical boundaries of the PVN (scale bars = 100μm). The locations of the immunoreactive elements suggest that these proteins may be expressed in some of the same cells.

$GABA_BR1$ subunit knockouts exhibited a decrease in BDNF expression.

BDNF protein is expressed throughout the PVN as early as E15 and through adulthood (data not shown). At E17, $GABA_BR1$ subunit knockout mice had decreased BDNF immunoreactivity in the PVN in a region dependent manner (Fig. 7). Tissue processed for ir-BDNF in $GABA_BR1$ subunit wildtype and knockout animals was normalized for variability in background levels and was analyzed based on a grid analysis. All boxes were determined as being a part of the PVN or outside of the region of the PVN. The data were analyzed as immunoreactive area in a two-way ANOVA, genotype by column location considered as a repeated measure. Calculated by total immunoreactive area over all columns, the knockouts exhibited a 32% decrease in immunoreactive area. The analysis showed a significant

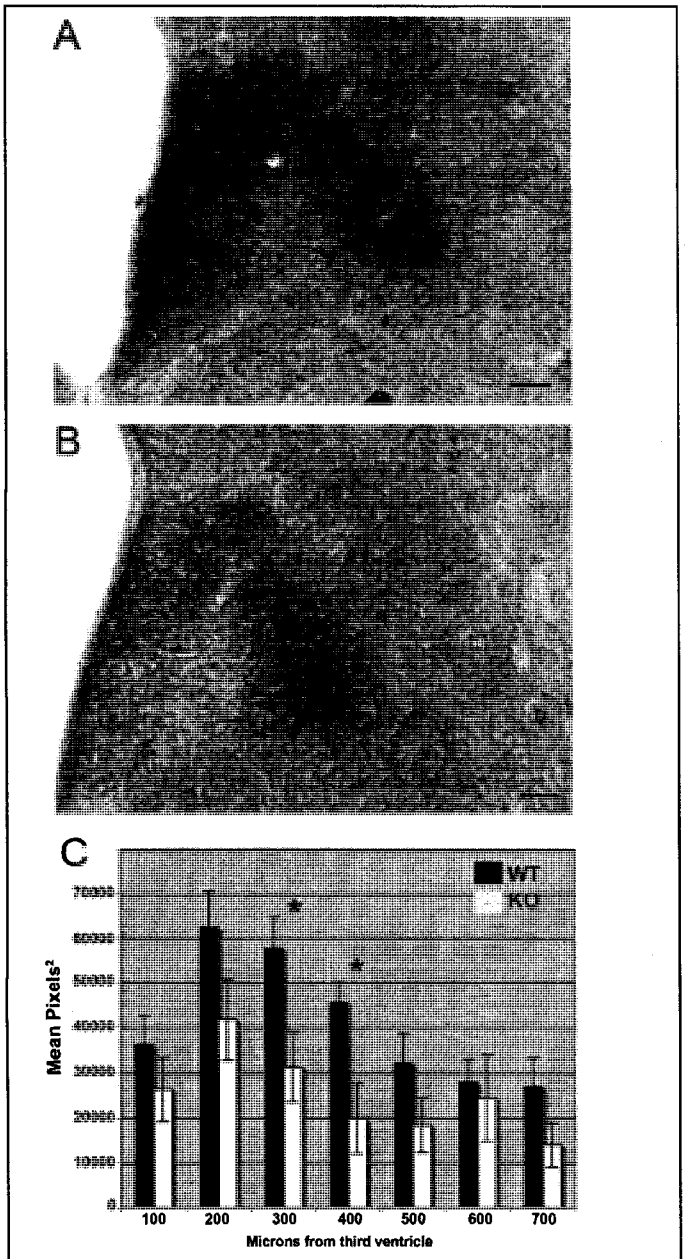

Figure 7 – $GABA_BR1$ subunit knockout mice (B, n=5) had a 32% decrease in immunoreactive BDNF in the PVN compared to wildtype mice (A, n=7) with a significant difference between genotypes ($p<0.05$). Analyzing the immunoreactivity by location revealed that the largest decrease in protein levels was between 300 and 500μm from the edge of the third ventricle (C, scale bars =100μm).

between genotypes effect ($F_{(1,9)}$=5.93, $p < 0.05$). While the genotype by column location interaction was not statistically significant, those columns in the central region of the PVN (300-500μm from the edge of the third ventricle) were the only columns that had mean values outside the 95% confidence intervals for genotype comparisons. The VMN is another site rich in BDNF (Connor et al., 1997) and where $GABA_B$ receptors play a role in nuclear development (Davis et al., 2002; McCellan et al., 2008). A similar analysis of ir-BDNF in the VMN of $GABA_BR1$ subunit knockout mice showed no discernible change in the levels of immunoreactivity (data not shown).

Discussion

The development of the hypothalamus involves the interactions of a combination of many factors, including transcription factors, secreted factors, cell signaling molecules and extracellular matrix proteins to accomplish the goal of forming and connecting key components of specific nuclear groups. At E15, in the mouse hypothalamus, the PVN is not discernible in Nissl stained coronal sections. By contrast, tissue sections immunoreactive for various molecular markers of the PVN demarcate the beginnings of a defined cell group. Terminal differentiation of cell fate can partly be determined by the production of specific hormones such as corticotropin releasing hormone (CRH), vasopressin, and oxytocin that are found relatively early (around E15) in the developing PVN (Michaud et al., 1998b). In the current study, these and other molecular markers such as BDNF, calbindin, neuronal nitric oxide synthase, ERα and β, galanin, and NPY help further characterize the peptide and protein expression profile of the embryonic PVN. These markers, and others (Caqueret et al., 2006), illustrate that an organized and heterogeneous nucleus is apparent before the PVN becomes a cell group discernible by Nissl stain. At least 2 of these factors have been shown to either synthesize a molecule (nNOS catalyzing the synthesis of nitric oxide; (Bicker, 2005)) or

act itself (BDNF) to alter cell migration (Behar et al., 1997; Borghesani et al., 2002). Furthermore, similar to the VMN, elements containing immunoreactive GABA surround the region that becomes the PVN, and GABA may play key roles in determining cell positions in hypothalamus (Tobet et al., 1999).

GAD and GABA are found in cells and fibers throughout the hypothalamus at early ages. Before the establishment of axonal connections, GABA is not likely acting in its traditional role as a neurotransmitter at the level of synapses. The functional significance of GABA in early development may be to influence developmental processes ranging from proliferation, migration, and differentiation (Lauder, 1993; Nguyen et al., 2001). In the hypothalamus, two nuclear groups form, for which GABA synthesis does not take place within the cells and fibers of the nuclei themselves – the PVN and the previously studied VMN (McClellan et al., 2006). The current data shows that cells and fibers synthesizing GABA surround the developing PVN as early as E13, and that cells within the region of the developing PVN have $GABA_B$ receptor subunits (R1).

Estrogen receptors in vertebrate brains play important roles during development and in adulthood. Studies over the last decade suggest that ERα is expressed in some neurons early in development. In mice, the number of hypothalamic cells containing ir-ERα increases from E13 to adulthood in rostral-caudal and medial-lateral dimensions. It is a particularly illuminating cell phenotype to examine from the perspective of cell positions (Tobet et al., 2002). The role of GABA in VMN development has been studied using a combination of GABA receptor subunit knockout mice (Dellovade et al., 2001), as well as imaging cells from live slice preparations (Davis et al., 2002; McClellan et al., 2008). The current study extended the examination of estrogen receptors in development to

cells containing ir-ERα within and around the PVN. The PVN contained a small population of ERα positive cells along the third ventricle and the dorsal region of the rostral PVN with a larger population of ERα positive cells lateral to the PVN. The current study utilized $GABA_B$ R1 subunit knockout mice (Prosser et al., 2001) to look at ir-ERα in the absence of $GABA_B$ receptor signaling. In female, but not male, knockout mice there were fewer immunoreactive cells closer to the third ventricle and more cells grouped lateral to the PVN. In the absence of a significant alteration in the total number of ERα immunoreactive cells in the region as a whole, this difference may reflect an influence on cell migration.

The pattern of ERα expression in the adult PVN is largely periventricular and dorsal and ventral to the PVN in the rat (Suzuki and Handa, 2005) and somewhat similarly in the mouse (e.g., figure 5C). The lateral population of ERα cells that was altered in P0 female $GABA_B$ R1 subunit knockout mice may be part of the lateral hypothalamic/ perifornical region of the hypothalamus. The perifornical region is part of the hypothalamic area controlling emotional responses (HACER) in the primate (Smith et al., 1990) and is involved in the regulation of cardiovascular responses to emotions (Risold et al., 1994). The perifornical population of cells within the hypothalamus has been implicated in aggressive behaviors and autonomic cardiovascular responses and contains a large number of orexin positive cell bodies (Peyron et al., 1998; Steininger et al., 2004). These behaviors can be responsive to hormones, therefore it is possible that the ERα cell population lateral to the PVN are at least a part of the hormone responsive perifornical region of the hypothalamus. In the context of the current results, we reexamined the pattern of ERα immunoreactivity in the PVN region of $GABA_A$ receptor β3 subunit knockout mice (Dellovade et al., 2001) and found no changes. However, the

$GABA_A$ receptor β3 subunit is expressed at extremely low levels if at all in the embryonic PVN (data not shown), therefore we can not conclude that $GABA_A$ receptors do not affect the development of the PVN as other subunits (including high expression of the epsilon subunit; (Tobet et al., 1999) are expressed in this region.

There are a number of indications of sex differences in PVN regulation and function (Handa et al., 1994; Rhodes and Rubin, 1999), even though there are relatively few findings of sexual dimorphism (differences based on cytoarchitecture and anatomy) in the adult or neonatal PVN. One recent finding is a difference in the number of CRH neurons in the brains of human subjects. Men had more CRH neurons than women in the PVN and men, but not women, had a significant increase in the number of CRH neurons with age (Bao and Swaab, 2007). The data presented here suggests a sex difference in the positioning of cells containing ir-ERα that was only revealed when $GABA_B$ receptor signaling was impaired. Many sex differences in the PVN may only become apparent under specific physiological states or in circumstances of altered development.

In the current study, a sex difference emerged in a region lateral to the classical border of the PVN. It is important to consider the relationship of the cells in any nuclear group to those in neighboring regions (Gahr, 1997). For the VMN, the neighboring 'tuberal' nucleus was defined as a lateral component of the VMN based on similarities and differences of cellular characteristics and projections (Canteras et al., 1994). For the PVN, accessory magnocellular nuclei (Mathieson et al., 2000) may be reminders or remnants of developmental migrations (Ugrumov, 2002). Developmental influences of gonadal steroids (Knoll et al., 2007; McCarthy, 2008) or sex chromosomes (Arnold et al., 2004; Budefeld et al., 2008) may impact the relative ability of cells in or around to be part

of the classical nuclear configuration or connected merely by similar function. These neighboring cells may also be a part of a population of neurons that projects to the PVN. Inputs from forebrain and hypothalamic structures are directed primarily to nonmagnocellular subregions. These inputs to the PVN arise from the anteroventral periventricular nucleus, arcuate nucleus, lateral hypothalamic area, preoptic area, dorsomedial nucleus, and the periventricular area (Sawchenko and Swanson, 1983). Of particular interest are the inputs from the lateral hypothalamic area that occupy the area just lateral to the PVN.

In the current study, there were more cells with multiple nucleoli along the medial zone of the PVN, less in the lateral region, and an apparent increase in cells outside of the PVN. Furthermore, there were fewer cells with multiple nucleoli at older ages in agreement with previous results in the VMN (Tobet et al., 1999). There are currently 3 major functions attributed to nucleoli; rRNA editing, RNA splicing, and ribosomal biogenesis (Boisvert et al., 2007). One potentially important component located within the nucleoli is small nucleolar RNA's (snoRNA) that are a group of non-coding RNA's involved in RNA modification; mostly ribosomal, but also potentially messenger. The presence of multiple nucleoli at specific points in development (or locations) may indicate that significantly greater levels of RNA editing are occurring. Two snoRNA sequences found in mouse brain (MII-52 and MII-85; (Huttenhofer et al., 2001) have been implicated in Praeder-Willi syndrome (Sahoo et al., 2008), a hypothalamic disorder characterized by abnormal growth, cognitive deficits, and hyperphagia resulting in obesity. A mouse model knockout for MII-52 has a phenotype similar to other Prader-Willi mouse models (Ding et al., 2005). The human paralogue to the mouse MII-85 gene is found within the chromosomal region that is affected in Prader-Willi patients. The paraventricular nucleus is one site in the brain that has been suggested as a locus for

the Prader-Willi phenotype (Gabreels et al., 1998; Swaab et al., 1995). Based on the importance of snoRNA's for a particular hypothalamic disorder as well as the differences in RNA editing in development, the presence of multiple nucleoli in the cells of the developing PVN may correspond to changes in RNA editing that ultimately effect gene transcription.

BDNF is a neurotrophic factor that has characterized roles in neuronal growth, migration, survival and differentiation (Chiaramello et al., 2007; Fukumitsu et al., 2006; Gorski et al., 2003; Turner et al., 2006). BDNF is synthesized in specified locations within the embryonic hypothalamus, including a concentrated group of cells synthesizing BDNF in the PVN (Tapia-Arancibia et al., 2004). There are several ways to consider BDNF in the developing PVN; one from the perspective of a factor that influences PVN development (e.g., migration or cell survival), two, as a factor that determines PVN function (e.g., synaptic alteration in CRH secretion; (Givalois et al., 2001), or three, as an indicator of PVN differentiation (e.g., cells in position with fate chosen). In the current study, BDNF was found in the PVN by E15, in time to play a role in PVN development and differentiation or to report on the influence of other factors. By E17, in the $GABA_BR1$ subunit knockout mice, there was a region-specific decrease in ir-BDNF within the PVN. The central part of the PVN is where the most obvious decrease in immunoreactive BDNF was seen. Although, the phenotype of these cells remains to be determined, the decrease in the central PVN indicates that only selected cells within the PVN are affected by a loss in $GABA_B$ receptor signaling. $GABA_B$ signaling could contribute to either the terminal differentiation or cell positioning of cells that are dependent on GABA. In other systems, $GABA_B$ agonist in hippocampal cells increased BDNF expression (Ghorbel et al., 2005), and in embryonic hypothalamic cultures GABA increased the expression of BDNF through the MAPK and CREB pathway (Obrietan et al., 2002).

Thus, GABA signaling may be involved in the differentiation of neurons synthesizing BDNF and the disruption of GABA signaling through the deletion of the $GABA_B$ receptor may influence the differentiation of BDNF cells within the PVN.

It is clear that not all cells expressing BDNF in the brain are affected by $GABA_B$ receptor signaling. In the current results, BDNF expression was only reduced in central portions of the PVN. In the VMN, there were no differences in BDNF mRNA (McClellan et al., 2008) or protein (current study) in $GABA_BR1$ knockout mice. BDNF regulation could be different based upon the neurochemistry of each cell type. One method of rapid modulation of BDNF levels in adult animals is through increased stress (Rage et al., 2002). However, stressful stimulation during pregnancy has also been shown to increase maternal and fetal neuronal activity and fetal BDNF protein in the PVN (Fujioka et al., 2003). It could be that neuronal subtypes within the PVN involved in the stress response are more susceptible to changes in BDNF levels. Because BDNF regulation may differ within nuclei located nearly adjacent to one another, to study such a system it will be necessary to develop and utilize methods with better cellular resolution.

The PVN is the final regulator in the feedback pathway that ultimately controls the hypothalamo-pituitary-adrenal (HPA) axis and the hormonal response to stress. Anxiety-like and depressive-like behaviors in animal models result from manipulating the HPA axis and increasing circulating levels of CRH (Kasckow et al., 2001). The importance of the PVN and the HPA axis as it relates to anxiety-related disorders has also been shown in human studies. Understanding normal development of the PVN and which factors contribute to its development can help us further understand this nuclear group and how abnormal development could ultimately lead to altered function and dysregulation of the HPA axis. The results of the current study suggest that GABA plays a role in setting up

signals for neurons moving in the region of the PVN. In the absence of a functional $GABA_BR1$ subunit cell positions and protein expression were altered in and around the PVN. A second $GABA_BR1$ subunit knockout mouse, generated by a separate group, was tested for behavior changes in adulthood. These mice exhibit behaviors that suggest they have decreased anxiety as compared to their littermate counterparts (Mombereau et al., 2004; Schuler et al., 2001). BDNF function in adulthood has been linked to depression and other mood disorders (Angelucci et al., 2005; Hashimoto et al., 2004), although less has been done developmentally. However, the recent findings of depression in forebrain selective murine knockouts of BDNF (Chan et al., 2006; Monteggia et al., 2007) suggest that there may be significant developmental contributions of BDNF to adult depression. Interestingly, disruption of the transcription factor SF-1 alters the differentiation of the VMN (McClellan et al., 2006) including causing a site-specific decrease in BDNF expression and an increase in anxiety-like behavior (Zhao et al., 2008). Changes in anxiety-like or depression related behaviors subsequent to $GABA_B$ receptor inactivation may be related to HPA axis regulation and the PVN.

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Chapter 5

Conclusions and Discussion

There are 5 major processes through which the development of the hypothalamus, as well as other aspects of the CNS, occurs. These processes include cell proliferation/ neurogenesis, cell migration, cell death, fate determination/ differentiation, and axonal projections to make synaptic connections. This discussion will focus on the development of the VMN, PVN, and ARC pertaining to these five processes and how GABA signaling may play roles in development relative to these categories. Much of this information has been discussed in chapter 2 in regards to the VMN, however, it has also been included in this discussion for comparison to other regions. The focus of this thesis is an examination of the role(s) GABA plays in the development of hypothalamic nuclear groups.

Following the divisions of progenitor cells along the proliferative zone of the third ventricle, developing neuroblasts must migrate to form distinct hypothalamic cell groups. Neurons migrate radially, away from ventricular zones, guided by radial (glial) cell processes and tangential to such fibers, often along neuronal processes (Rakic et al., 1994). Radial glial fibers extend from the third ventricle to the surface of the brain in a dorsomedial to ventrolateral direction. The hypothesized pattern of migration is from dorsomedial to ventrolateral (Altman and Bayer, 1986) matching the pattern of radial glial fibers known to cross the VMN, PVN, and ARC (Dellovade et al., 2001; Levitt and Rakic, 1980; Tobet and Fox, 1989). Based on live video microscopy studies, migration can also occur in a dorsal ventral orientation, tangential to radial fibers, within the region

of the VMN and ARC (chapter 2; (McClellan et al., 2006). In contrast to the inside-out pattern of the cortex, the earliest born cells in the hypothalamus migrate the farthest from the ventricle.

GABA signaling alters cell movements and positions in the developing VMN and PVN. Based on previous data (Tobet et al., 1999), chapters 2 and 3 take a closer look at how the pattern of GABA contained within elements surrounding the VMN may provide an influence for its development. Chapter 4 describes how the same pattern of GABA surrounding a developing region was found for the PVN. At E13, four days before there is a nuclear group as defined by the Nissl-stained cytoarchitecture, there is a dense pattern of GAD/ GABA immunoreactivity in locations surrounding the developing PVN. The central hypothesis of chapter 4 is that the influence of GABA on development of the PVN would be similar to that in the VMN where GABA presumably influences cell migration. GABAergic cells and fibers are found throughout the brain at embryonic ages and are particularly dense throughout the hypothalamus. The PVN and VMN were the only regions within the hypothalamus where this pattern of immunoreactive GABA was observed. The ARC is one nucleus with GABAergic cells and fibers that are by contrast, integral to the nucleus, and therefore served as an interesting site to compare effects among nuclei. There were no discernible influences upon the addition of GABA receptor antagonists to hypothalamic slices containing fluorescently labeled cells in the region of the ARC (chapter 4; McClellan et al., 2008).

Five lines of evidence suggest that GABA plays a role in the embryonic differentiation of at least a subset of the cells in the VMN and PVN. GABA is synthesized in positions to provide potential boundary information for the embryonic VMN (Tobet et al., 1999) and PVN (chapter 4; McClellan and Tobet, submitted). Disrupting the SF-1 gene disrupts the

distribution of GABAergic elements in and around the VMN prior to its organization (Dellovade et al., 2000). Activation of $GABA_A$ receptors affects cell movements and the distribution of identified cells in the region of the VMN (Dellovade et al., 2001), while inhibition of $GABA_A$ receptors increases cell movement speed in the VMN (chapter 3; McClellan et al., 2008). Activation of $GABA_B$ receptors decreased cell movements in the region of the VMN (Davis et al., 2002), and antagonizing $GABA_B$ receptors increased cell movements (chapter 3; McClellan et al., 2008). Finally, disruption of $GABA_B$ receptor signaling affects cell positions in the developing VMN (chapter 4; McClellan et al., 2008) and PVN (chapter 4; McClellan and Tobet, submitted). Since the two receptors may be differentially sensitive to GABA levels (Johnson and North, 1992), the interaction of the two mechanisms may be spatially regulated by the locations of different concentrations of GABA (e.g., high at the nuclear boundary).

GABA can act through two receptor subtypes that utilize different mechanisms, however, they resulted in the same effect on cell motions. $GABA_B$ receptors are metabotropic G-protein coupled receptors (Gi/o) that activate cGMP-signaling pathways, activating potassium channels and altering calcium currents, changing the calcium concentration within cells (Bowery and Brown, 1997). These receptors are different in both structure and mechanism from $GABA_A$ receptors, which signal through the movement of chloride along its concentration gradient, changing the membrane potential of cells. However, since chloride moves outward in embryonic neurons causing depolarization and potential calcium entry (Gao and van den Pol, 2000), one possible common mechanism through which GABA may be influencing cell speed is through changes in intracellular calcium levels. The location of $GABA_A$ and $GABA_B$ receptors on the same cells in hypothalamic neurons has been shown to influence the behavior of the neurons. $GABA_B$ receptor mediated inhibition has been shown to affect $GABA_A$ receptor mediated calcium spikes

in embryonic hypothalamic neurons (Obrietan and van den Pol, 1998). There was no additive effect of sequential addition of $GABA_A$ and $GABA_B$ antagonists on movements within the VMN suggesting that the influence on motion was through a common mechanism. Activation of GABA receptors may also lead to changes in migration through different signaling pathways. Radial migration occurs throughout the nucleus, however, a neuron can detach from a radial fiber and move tangentially to a specific location along the lateral and medial edges of the nucleus (chapter 2; McClellan et al., 2006). Another possible role for GABA in migration is through this interaction between neurons and fibers. GABA may alter neuron/fiber interactions by influencing the synthesis or modification of adhesion molecules like PSA-NCAM (Lakhanpal and Kaur, 2007; Parkash and Kaur, 2007).

Magnocellular PVN neurons derive from progenitor cells that likely also give rise to neurons of the SON and distributed accessory nuclei (Ugrumov, 2002). Based on immunoreactive AVP, some of the magnocellular cells of the SON have reached their positions by E15 (Chapter 4 of the current thesis and Michaud et al., 1998; Cacqueret et al., 2006; Xu and Fan, 2007). The path from the PVN to the SON contains very few cells immunoreactive for AVP at early ages (at E15, very few AVP-immunopositive cells are not localized to the PVN or SON). The data suggests that the process of migration occurs early in development, before E15, and that to leave the boundaries of the PVN, certain neurons must move towards the GABAergic cells and fibers located outside the region of the developing nucleus. Portions of magnocellular neurons born between E10 and E12 in rats migrate laterally to the region just above the optic nerve to form the SON. The remaining neurons stay near the ventricle to form the PVN, along with later born parvocellular neurons. The cues controlling this partitioning of cells occupying the PVN versus SON are not well understood but indicate that proliferating cells along the

third ventricle must choose to either stay within the PVN, or move towards a region outside of the PVN, regardless of having a similar phenotype (e.g. vasopressin and ERα). ERα cells migrating through the PVN either stay within the periventricular region of the PVN, the dorsal cap of the PVN, or move outside of the PVN towards the lateral hypothalamic area. These cells must respond differently to the GABA signal surrounding them, since a subset actually move towards GABA, and are therefore, not being influenced by the boundaries of the developing PVN (chapter 4; McClellan and Tobet submitted).

In other regions of the hypothalamus, including the ARC, cells move into and through regions containing GABAergic cells and fibers. The movement patterns of these cells are not influenced by the disruption of GABA signaling (chapter 3; (McClellan et al., 2008). This may be caused by a desensitization to GABA as many neurons in the ARC either synthesize GABA themselves or are surrounded by fibers and neurons that synthesize GABA (Behar et al., 1996; Gonzalez-Maeso et al., 2003).

GABA is one of a short list of secreted factors that may play a role in migration within the developing hypothalamus – other migratory influences on normal development of the VMN, PVN, and ARC are known at the level of transcription factors.

There is significantly more information about transcription factors whose functional presence is important for hypothalamic development. SF-1 exerts a large influence on VMN development by influencing the positions of cells in the region of the VMN, presumably in part by influencing the abilities of some cells to move. Interestingly, some cells born relatively late (E13) are more affected by a loss of SF-1 than cells born earlier (E11) based on a change in the position of BrdU positive cells within the VMN in SF-1

knockout mice (Davis et al., 2004a). Groups of phenotypically identified cells are "misplaced" or have an increased scatter pattern in SF-1 null mice.

Otp, Sim1, and Arnt2 are the key transcription factors thought to play a role in migration within the PVN. Otp mutants generated by a knocked-in LacZ allele had a significant reduction in cells containing detectable LacZ and these cells were found at lateral positions between the PVN and SON, not associated with either cell group. Sim1 and Arnt2 act as dimer partners in proliferation and migration of progenitor cells of the PVN/SON (Hosoya et al., 2001; Michaud et al., 1998). Sim1 expression may also influence cell migration within the region of the PVN (Xu and Fan, 2007). The distribution of Sim1 presumptive cells in the mutant indicates an altered migratory phenotype that may be mediated through the direct regulation of PlexinC1 (a receptor known to be involved in migration and axon guidance) by Sim1 (Xu and Fan, 2007). In the case of PlexinC1, little is known about the ligand semaphorin 3a in the PVN region. If PlexinC1 is mediating an altered migratory phenotype, the ligand to this receptor should be present in cells within the PVN.

The process of developing a functional nucleus begins with the "birth" or terminal mitotic division for neurons that populate the region.

Based on ^{3}H-thymidine incorporation studies, cells in the VMN derive primarily from precursors in the proliferative zone surrounding the lower portion of the third ventricle dorsal to the arcuate nucleus (Altman and Bayer, 1986). Neurons that populate the VMN are born between E10 and E15 in mice, E13 to E17 in rats, and around E30 in the primate (Shimada and Nakamura, 1973; Tran et al., 2003; van Eerdenburg and Rakic, 1994). Despite having been shown to be important in proliferation and development of adrenals and gonads (Bland et al., 2000), it is unclear whether SF-1 also has a role in

neurogenesis within the hypothalamus. In one report, SF-1 was not seen in BrdU immunoreactive cells that were mitotically active along the proliferative zone (Tran et al., 2003). In contrast, another study has shown that SF-1 is expressed in cells of the diencephalon as early as E9.5, when virtually all cells are mitotically active (Ikeda et al., 2001). Another receptor that may play a role in neuronal proliferation is the calcitonin receptor, specifically isoform 1a. Cells immunoreactive for this receptor are found at embryonic ages along the ventricular zone of the VMN as well as in other brain and hypothalamic regions where proliferation is occurring (Tolcos et al, 2003). A zinc-finger protein Zac1 was highly localized to the ventricular zone of the hypothalamus and in the region of the VMN. This protein plays a role in regulating cell cycle and has been hypothesized to be playing a developmental role in neurogenesis and inhibiting cell death in newly generated neurons (Valente et al., 2001).

The development of the PVN has been studied using Nissl stains, neuronal birthdating, and the identification of cell phenotypes at early ages. Based on Nissl staining, the PVN is first visible as a cell group between E15 and E17 in mice (Karim and Sloper, 1980; Shimada and Nakamura, 1973). Cells of the PVN are derived from precursors along the third ventricle with distinct timelines; more lateral (magnocellular) cells are primarily born between E10.5 and E12.5 in the developing rat (Altman and Bayer, 1986; Markakis and Swanson, 1997), with medial (parvocellular) cells being born later. Otp is one transcription factor involved in the control of proliferation, survival, and migration of PVN/SON progenitors (Acampora et al., 1999; Wang et al., 2000). Otp mutants generated by a knocked-in LacZ allele had a significant reduction in cells containing detectable LacZ. Sim1 was thought to play a role in proliferation but more recently, a report showed that bromodeoxyuridine incorporation were similar in Sim1 mutants compared to wildtype mice (Xu and Fan, 2007) suggesting no effect on cell proliferation.

Less is known about factors that may be involved in cell proliferation for the region of the ARC. The calcitonin receptor, and Zac1 as described above for the VMN have also been implicated in the proliferation of cells along the ventricular zone of the ARC. This seems plausible with the close proximity of these two nuclear groups. Video microscopy analyses in brain slices from embryonic mice indicate that cells populating the region of the ARC and the VMN are moving away from the third ventricle at similar locations with ARC neurons ventral to those headed toward the VMN (chapter 3, Fig. 1). Aside from these factors one other molecule studied in depth as it relates to the development of the ARC as a whole is leptin. There is some suggestion that leptin plays a role in proliferation, however, most of the research regarding leptin's role in development is through its role in establishing functional feeding circuitry within the ARC, PVN, and dorsomedial hypothalamus (Bouret and Simerly, 2004).

In the developing brain, programmed cell death plays a role in the organization of brain structures.

Almost 10 years ago it was realized that there may be significantly more cell death during normal development than previously appreciated (Blaschke et al., 1996). Subsequent experiments revealed significant cell death in regions of the hypothalamus that are sexually dimorphic (Arai et al., 1996; Davis et al., 1996; McCarthy et al., 1997). Sex differences in cell death have emerged as a central component of many theories of brain sexual differentiation (Forger et al., 2004). In rats, there was very little apoptosis during postnatal development of the VMN. As the nucleus increased in size from PN2 – PN12, the amount of cell death decreased (Chung et al., 2000). Based on the locations of dying cells, there does not appear to be a major role for cell death in the emergence of the VMN (McClellan et al., 2006). Across all 3 nuclei in general it did not appear that

cell death played a major role in the development of the VMN, PVN, or ARC. Pyknotic cells (an indicator of cellular compaction as an endpoint to a dying process that could be necrotic or apoptotic in the developing brain) were counted in VMN sections and no more than 4 cells were found per each 50μm thick section (Davis et al., 2004a). Preliminary studies examined immunoreactive activated caspase 3 as a widely used marker of apoptotic cells. At P0 in the VMN there were very few cells containing this marker in mice (2-4 cells/ 50μm thick section). The PVN had more activated caspase 3 positive cells (5-8 cells/ 50μm thick section) than found in the VMN. Another group (Chung et al., 2000) also found an increase, as compared to other nuclear groups, in cell death within the PVN throughout development. The ARC numbers closely resembled those of the VMN.

BDNF plays an important role in cell survival and cell migration within the developing hypothalamus.

In conjunction with cell death, cell survival is also an important part of the development of any brain structure. One growth factor – brain-derived growth factor (BDNF) – has a stereotyped and highly regionalized expression pattern in the developing mouse hypothalamus. BDNF is found early in development within the VMN, PVN, and in smaller amounts in the ARC. It is a key neurotrophic factor controlling diverse brain functions in development and in adulthood. BDNF is a member of the neurotrophin family of proteins with activity mediated by binding to a specific receptor tyrosine kinase (TrkB) as well as the non-selective receptor $p75^{LNTR}$ (Tapia-Arancibia et al., 2004). In the rat, BDNF expression is high from birth through P6, followed by a rapid decrease in BDNF expression from P6 through adulthood (Sugiyama et al., 2003). BDNF has been shown to play a role in the migration of cerebellar granule cells as well as cortical neurons thus demonstrating potential chemokinetic and chemotactic roles for BDNF (Behar et al.,

1997; Borghesani et al., 2002). However, when brains from BDNF knockout mice (generously provided by Dr. R.A. Segal, Harvard University, Boston, MA) were examined for changes in VMN cell positioning, there was no obvious alterations in cell positions suggesting that the role BDNF plays in the development of the VMN relates to physiological function(s) (Tran et al., 2006; Zhou et al., 2008) and does not involve cell migration. Another group also examined the BDNF knockout mice for changes in the VMN based on selected mRNA levels in development. After looking at numerous VMN markers, they did not find differences at P0 (SfN abstract; Holm et al., 2007). These data taken together suggest that BDNF may be important for cell survival but does not play a role in migration within the region of the VMN.

The PVN contains high levels of BDNF and TrkB mRNA (Givalois et al., 2004). We have shown that $GABA_BR1$ subunit knockouts exhibit a selective decrease in BDNF expression in the PVN leading to the hypothesis that GABA and BDNF may act synergistically to determine aspects of PVN development. There are several ways to consider BDNF in the developing PVN; one from the perspective of a factor that influences PVN development (e.g., migration or cell survival), two, as a factor that determines PVN function (e.g., synaptic alteration in CRH secretion; (Givalois et al., 2001), or three, as an indicator of PVN differentiation (e.g., cells in position with fate chosen). Immunoreactive BDNF was found in the PVN by E15 (Chapter 4; McClellan and Tobet, submitted), a time point important in PVN development and differentiation. By E17, in the $GABA_BR1$ subunit knockout mice, there was a region-specific decrease in immunoreactive BDNF within the PVN. In prenatal hypothalamic neurons, BDNF influenced the release of GABA, while in turn, GABA release stimulated BDNF expression (Obrietan et al., 2002). $GABA_B$ signaling could contribute to either the terminal differentiation or cell positioning of cells that are dependent on GABA. In other

systems, $GABA_B$ agonist in hippocampal cells increased BDNF expression (Ghorbel et al., 2005), and in embryonic hypothalamic cultures GABA increased the expression of BDNF through the MAPK and CREB pathway (Obrietan et al., 2002). Thus, GABA signaling may be involved in the differentiation of neurons synthesizing BDNF and the disruption of GABA signaling through the deletion of the $GABA_B$ receptor may influence the differentiation of BDNF cells within the PVN.

Morphological sex differences in the brain may underlie sex differences in function. In the developing mouse hypothalamus, few sex dimorphisms (anatomical characteristics/ cellular chemistry) have been found. On the other hand, numerous sex differences in function and response to stimuli are recorded throughout the literature. We found a difference in positioning of $GABA_BR1$ receptor subunits in the POA/AH at E17 both in protein and mRNA with females having $GABA_BR1$ located in more dorsal positions (Wolfe et al., 2005). The POA/AH of the mouse is a region known for sex differences in volume, neuron number, and fiber projections in many other vertebrates (Simerly, 2002; Tobet et al., 1993). A difference in the location of $GABA_B$ receptor subunits may result in changes of GABA signaling. We have not found a similar sex difference in the location of $GABA_B$ receptor subunits within the region of the VMN or PVN (data not shown). Therefore if there is a sex difference in $GABA_B$ function in the PVN (e.g., Chapter 4), it is likely due to a mechanism other than simply the starting positions of cells that express the subunits.

We found a sex-specific effect of ERα cell positions as a response to GABA signaling in the PVN (chapter 4; McClellan and Tobet, submitted). Female $GABA_BR1$ subunit knockout mice had an increase in ERα positive cells in the lateral hypothalamic area just outside the PVN, and a decrease in ERα cells within the PVN. Because the total amount

of immunoreactive ERα (as a proxy for cell number) does not change between genotypes, this phenotype is likely due to changes in cell migration. The lack of functional $GABA_B$ receptors is most likely the reason for these changes in cell positions, however, our lab has also shown that estradiol administration to a live slice can cause changes in movements (Knoll et al., 2007). This suggests the occurrence of changes in cell migration based on steroid hormone levels in development. One additional sex-specific difference found within the $GABA_BR1$ subunit knockout mouse colony was a body weight decrease at E17 and P0 in females only (graphs; appendix 1). Because the body weight difference is seen at prenatal ages, we can not conclude that this is the result in changes in feeding behavior, however, this is also well before the establishment of seizures, which is the explanation made for decreased body weight and appetite at weaning (Prosser et al., 2001). In chapter 3, we looked at cell positions at ages E17, P0, and P4. There was no sex difference in cell positions of the VMN at P0, but we could not examine all ages because generating P4 female knockout mice proved to be difficult. Of 8 litters with a combination of wild type, heterozygous, and homozygous knockout pups, there was only 1 female knockout found, in comparison to 12 male knockouts and 46 total pups. It may be that lack of functional $GABA_B$ receptors, in this line of mice, causes a decrease in intrauterine growth that results in mortality at young ages.

Sex-specific effects in development are particularly interesting as they relate to the relationship of the PVN to the regulation of the hypothalamo-pituitary-adrenal (HPA) axis. The PVN is the final regulator in the feedback pathway that ultimately controls the HPA axis and the hormonal response to stress. The HPA axis and our response to stress is a healthy and normal function associated with feedback mechanisms and autonomic responses, however, many individuals can have an abnormal and unhealthy response to stress. Depression has been called the most significant mental health risk

for women, especially younger women of childbearing and childrearing age (Glied & Kofman, 1995). Anxiety disorders are the most common mental illness in the U.S., affecting nearly 40 million adults (about 20% of the U.S. population). With most anxiety disorders, women are twice as likely to be affected than men (Anxiety Disorders Association of America statistics). A difference in gender susceptibility to anxiety may be closely related to sex differences in development, particularly in regions of the brain known to be involved in the regulation of anxiety and depression.

In conclusion, the data presented in these chapters better defines the role of GABA in the VMN and its influence on cell movements and cell positions. The data presented here also furthers our understanding of developmental roles for GABA in the CNS and in the hypothalamus in particular, as it relates to cell migration and cell fate determination. Many more answers are needed as we continue to learn more about the developing hypothalamus and in particular about how changes in the development of specific nuclei may influence changes in hypothalamic function.

Future Directions

1. To determine why cells containing ERα respond differently to GABA. The results from chapter 4 indicate that 1 population of neurons containing immunoreactive ERα moves towards GABAergic cells and fibers lateral to the PVN while another group stays within the boundaries of the PVN. These cell populations may provide a way to determine what other cues determine whether a cell recognizes GABA as an attractant or repulsive signal. It may be that cells in the lateral population of cells containing immunoreactive ERα are also producing nitric oxide while cells within the PVN at early ages are not making nitric oxide but are synthesizing BDNF and the calcium binding protein calbindin. These cells also may contain different GABA receptor subtypes. The ERα cell populations within the PVN are likely heterogeneous with different neurochemistry, and for that reason, respond differently to GABA.

2. To see if cell movements of the PVN respond as those in the VMN do. GABA administration to live slices caused an increase in cell movements in the region of the VMN. The data from chapter 4 implies that cells of the PVN may also be responsive to GABA receptor antagonists. Cell positions moved from more medial positions to more lateral positions indicating that the cells may increase their speed in response to altered GABA signaling.

3. What is the role of BDNF in the developing PVN? Does the decrease of BDNF synthesis cause a change in differentiation, cell survival? There was a decrease in BDNF protein levels in the $GABA_BR1$ knockout mice at E17. It would be interesting to see if this decrease was found at younger ages and if it affects the differentiation or survival of cells of the PVN. This could be done by looking at activated caspase 3 immunoreactivity as a marker for apoptotic cells. To further determine the role of BDNF in the PVN, utilization of the BDNF loxP mouse crossed to the Sim1 Cre mouse will eliminate BDNF expression only in areas with Sim1 expression, mainly the region of the PVN. Looking at the expression of PVN markers in these mice may reveal a role for BDNF in the development of the PVN. Furthermore, it has been shown that BDNF administration to the PVN alters feeding behavior. These mice would be a better model for testing the role of BDNF expression, in the PVN, to feeding behavior and anxiety.

4. Do misplaced ERα cells ultimately make the right connections or do they connect to alternate regions? What regions are projecting to these ERα cells? Do they look similar in terms of morphology and projection patterns? Do they have the same response to estrogen? Using transgenic mice can make it possible to answer some of these questions. Using a sim1-Cre transgenic mouse and crossing this with a loxP wheat-germ agglutinin reporter mouse, will give us a line of mice that we can look for immuonreactive wheat germ agglutinin in cells expressing Sim1. Sim1 is a transcription factor found in cells of the PVN, SON, and lateral hypothalamic regions adjacent to the PVN. Therefore, projections from PVN cells can be traced along their pathway by looking for immunoreactive wheat germ agglutinin. Using dual label fluorescence for ERα, this specific cell population can be identified and its projections located. Although this may not be

possible currently in the $GABA_BR1$ knockout line, the differences between the normal projection pathways of ERα cells occupying the dorsal and medial region of the PVN versus the projections of the lateral hypothalamic population of ERα cells could be found using the Sim1-Cre and loxP wheat-germ agglutinin reporter lines.

5. What is the extent of afferents and efferents relative to the PVN and VMN in fetal mice? Several reports indicate that the first projections from the ARC to the PVN develop postnatally in rats (Bouret and Simerly, 2004; Grove and Fukuchi-Shimogori, 2003). On the other hand, catecholaminergic fibers from the brainstem project to the PVN and ARC earlier in development, possibly before E15 in the mouse (unpublished observations; Grove and Fukuchi-Shimogori, 2003). Neurogenesis and migration in the PVN may occur earlier than in the ARC, therefore, connections from this nuclear group may be formed at different times in development. The loxP wheat germ-agglutinin mouse described above crossed with either the Sim1 Cre (PVN) or the SF-1 Cre (VMN), will generate lines of mice that when examined at different time points in development, may answer the question of when the earliest projections are being made.

6. Figure 1 of chapter 4 summarizes results regarding the number of cells containing more than one nucleolus. There were more cells with multiple nucleoli along the medial zone of the PVN, less in the lateral region, and an increase in cells with multiple nucleoli outside of the PVN. The amount of total cells with multiple nucleoli decreases throughout development with fewer found at later ages in both the VMN (Tobet et al., 1999) and PVN (chapter 4; McClellan and Tobet, submitted). With the function of the nucleolus as a site for RNA editing,

the presence of multiple nucleoli at specific locations or developmental time points may indicate that significantly greater levels of RNA editing are occurring. One potentially important component located within the nucleolus is small nucleolar RNA's (snoRNA), a group of non-coding RNA's involved in RNA modification; mostly ribosomal, but also potentially messenger. Seven brain specific snoRNA's have been described in mice (Hottenhoffer et al., 2001), one of which contains a conserved target recognition element that is a complementary match to the mRNA of the serotonin receptor 5HT2C (Kishore et al., 2006). A snoRNA without a target recognition element that corresponds to an RNA sequence has been called an "orphan". Two snoRNA sequences found in mouse brain (MII-52 and MII-85; Huttenhoffer et al., 2001) have been implicated in Praeder-Willi syndrome (for review, Peters et al., 2008), a hypothalamic disorder characterized by abnormal growth, cognitive deficits, and hyperphagia resulting in obesity. A mouse model knockout for MII-52 has a phenotype similar to other Prader-Willi mouse models (Ding et al., 2005; de los Santos et al., 2000). The human paralogue to the mouse MII-85 gene is found within the chromosomal region that is deleted in Prader-Willi patients. The paraventricular nucleus is one site in the brain that has been suggested as a locus for the Prader-Willi phenotype (Swaab et al., 1993; van Leuwen et al., 1995). Based on the importance of snoRNA's for a particular hypothalamic disorder as well as the differences in RNA editing in development, the presence of multiple nucleoli in the cells of the developing PVN may correspond to changes in RNA editing that ultimately effect gene transcription.

RNA editing may play an important role in developmentally regulating the functional properties of individual proteins. Results presented in chapter 4

indicate that the number of cells with multiple nucleoli is dependent on location and developmental age. Looking at changes in nucleoli within other regions of the hypothalamus and other ages including puberty and adulthood should answer the questions relating to number of nucleoli corresponding to maturity of the cells/ brain. The PVN is of particular interest as it relates to Prader-Willi syndrome, therefore it would be interesting to see if the two snoRNA sequences (MII-52 and MII-85) thought to play a role in PWS are particularly expressed at high levels in the PVN or any other regions of the hypothalamus. RNA probes to detect these specific sequences should be able to determine if all nucleoli contain snoRNA sequences or if they are location dependent.

ADAR2 is another editing enzyme located within the nucleolus, therefore, it would be interesting to determine if this enzyme is found in specific locations within the hypothalamus. Because the $GABA_A$ receptor subunit $\alpha 3$ is located within regions of the hypothalamus including the PVN and VMN, I would expect to see ADAR2 localized to these regions. It would be interesting to see if specific locations within these cell groups are undergoing more RNA editing in comparison to other regions.

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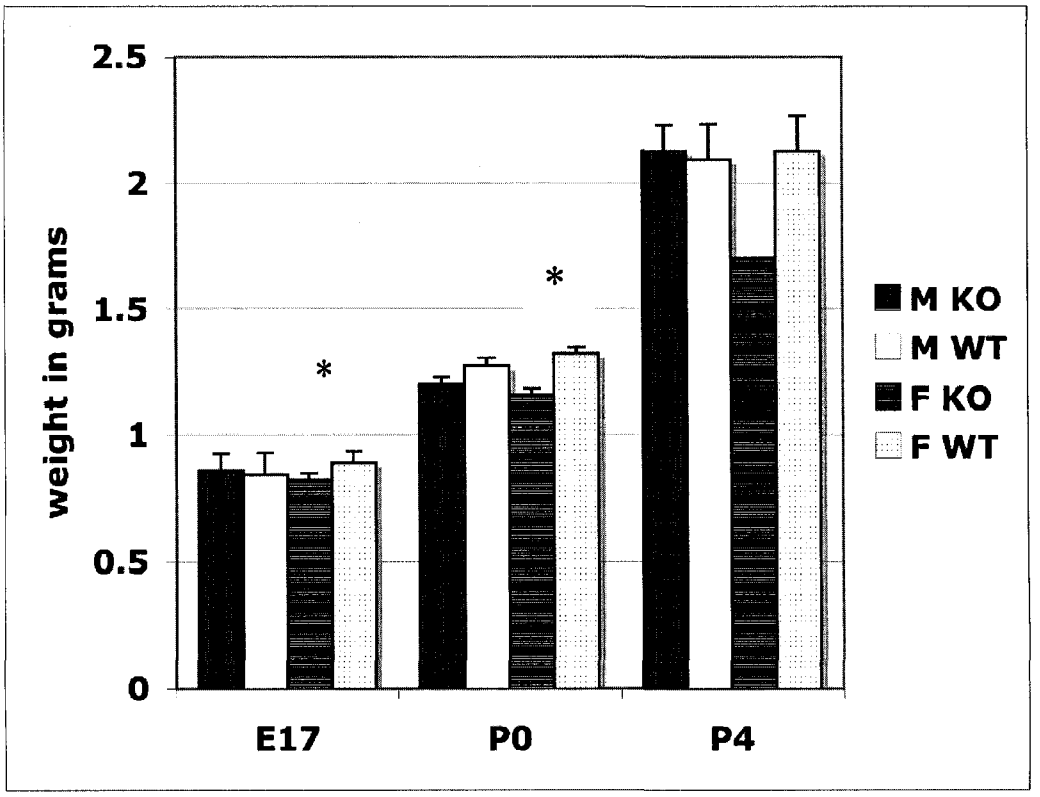
Appendix 1
Body weight analysis in $GABA_BR1$ knockout mice.



$GABA_BR1$ subunit knockout mice and wildtype littermates were perfused at ages embryonic day 17, postnatal day 0 and P4. At the time of perfusion, body weights were taken to verify developmental age. At E17 and P0, females had a significant decrease in body weight as compared to wild type littermates. This effect was not seen in males. At P4, only 1 knockout female was perfused therefore, we are not able to determine if there continues to be a significant decrease in body weight in females. At P4, males do not exhibit a decrease in body weight.