DISSERTATION

GAMMA-AMINOBUTYRIC ACID (GABA) IN THE DEVELOPMENT OF THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS (PVN): IMPLICATIONS FOR ADULT DISEASE

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ABSTRACT

GAMMA-AMINOBUTYRIC ACID (GABA) IN THE DEVELOPMENT OF THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS (PVN): IMPLICATIONS FOR ADULT DISEASE

The paraventricular nucleus of the hypothalamus (PVN) is the final common regulator of the neuroendocrine stress response. Humans with depression or anxiety disorders display altered regulation of this system and females are more likely to suffer from these disorders than males. This work investigated embryonic development of the PVN to identify cellular processes that might occur incorrectly (preferentially in females) and predispose the individual to altered regulation of stress responses. GABA acts as a neurotrophic factor during development. As the embryonic PVN is ringed by GABA (absence of GABA in the PVN) and a receptor for GABA is enriched in the PVN, it was hypothesized that this molecule would direct PVN development. Embryonic development was altered in mice either by genetic manipulation (receptor knockout mice) or by pharmacological blockade of the $GABA_A$ and $GABA_B$ receptors. Embryonic $GABA_A$ receptor antagonism caused a decrease in the number of neurons that expressed estrogen receptor α in and around the PVN. In female but not male mice lacking $GABA_B$ receptors, the cytoarchitecture of the PVN was altered. Specifically, estrogen receptor containing cells were misplaced and corticotropin releasing hormone was increased. Animals treated with a $GABA_B$ receptor antagonist during embryonic development copied the phenotype of receptor knockout mice. The *in vivo* effect of GABA signaling on cell placement was investigated *in vitro* with organotypic slice fluorescence video microscopy. Again only in females, blockade of the $GABA_B$ receptor caused neurons to increase migration speed. Thus GABA acts to restrict cells from moving outside of the PVN. When the $GABA_B$ receptor is antagonized, cells migrate outside of the PVN. To determine the consequence of an animal having altered PVN

development, animals treated as embryos with the $GABA_B$ receptor antagonist were subjected to a battery of behavior tests as adults. Interestingly, females treated embryonically with CGP 55845 displayed an increased anxiety-like phenotype (female specific disorder) while males treated with the same compound displayed a hyperactivity-like phenotype (male specific disorder). Independent of sex, animals treated as embryos with the $GABA_B$ receptor antagonist displayed decreased depression-like behaviors and had a less robust stress response compared to vehicle treated animals. This work highlights the importance of GABA signaling in PVN development and the dependence of complex adult behaviors on embryonic brain organization as GABA receptor antagonism limited to a specific critical time period during embryonic development recreated cytoarchitectural and behavioral phenotypes of GABA receptor knockout mice.

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CHAPTER 1. INTRODUCTION

PVN: Structure and Function

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 (e.g., corticotropin releasing hormone (CRH), arginine vasopressin (AVP), oxytocin (OT) Thyrotropin releasing hormone (TRH) and somatostatin (SS)); (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. Neuroendocrine neurons of the PVN project to either the median eminence where peptides are carried to the anterior pituitary by the portal vasculature or to the posterior pituitary where peptides are released directly into the circulation.

The amount of peptide required to be released at the posterior pituitary to be found at physiological concentrations in distant regions of the body (e.g., mammillary glands or kidneys) is greater than that required for the activation of cells in the anterior pituitary. Thus the cell bodies of neurons projecting to the posterior pituitary in most species are larger (magnocellular neurons) than the cell bodies of neurons projecting to the median eminence (parvocellular neurons).

In addition to regulating neuroendocrine function the PVN also has projections that remain within the central nervous system. These include projections to brain stem nuclei to regulate

autonomic function and the spinal cord (Toth et al., 1999 and Geerling et al., 2010), projections to other hypothalamic nuclei, and projections to limbic centers that translate information to and from the cortex.

The highly interconnected nature of hypothalamic nuclei is an important trait that is conserved across species (Swanson and Sawchenko, 1983). It would be inappropriate for an animal escaping a predator to stop to either eat or engage in mating behaviors. Conversely, if an animal were in a safe environment, with ample food and possible mates, yet unable to dampen its stress response, it would be unlikely to contribute to the next generation. The PVN integrates threat, hormonal, emotional, physiological and cognitive information throughout the lifespan to regulate appropriate 1) autonomic responses (Ulrich-Lai and Herman, 2009), 2) neuroendocrine stress responses (Saffran et al., 1955), 3) blood pressure maintenance (Andersson et al., 1975), 4) metabolism (Aizawa and Greer, 1981), 5) growth (Epelbaum 1986), 6) sexual function and lactation (Sofroniew 1983), 7) body temperature (Takahashi et al., 2001), 8) appetite (Kalra et al., 1999), and 9) arousal (Sato-Suzuki et al., 2002).

Cytoarchitecture:

The PVN has been broken down into ten distinguishable subdivisions in the rat. In the mouse PVN, magnocellular and parvocellular compartments overlap with one another, which has been an obstacle for defining key features of mouse PVN cytoarchitecture. A detailed description of mouse PVN cytoarchitecture in comparison with the rat has recently been published (Biag et al., 2012). Below is a description of mouse PVN cytoarchitecture, identifying specific compartments when appropriate. Generally, rostral portions of the PVN contribute to neuroendocrine regulation through the pituitary while neurons found caudally

project centrally (and usually caudally to brainstem and spinal cord sites) and function as autonomic regulators.

Within the magnocellular neuroendocrine division, anterior, medial, posterior medial and posterior lateral zones have been categorized. The anterior zone is comprised mainly of magnocellular OT containing neurons just caudal to the crossing of the anterior commissure and dorsal to the medial preoptic area. More caudally the medial magnocellular compartment is comprised mostly of OT containing neurons. Behind this, both OT and AVP containing magnocellular neurons are found in the posterior medial magnocellular compartment. In more caudal regions, the number of OT containing neurons decreases while the number of AVP containing neurons increases.

Parvocellular compartments are broken into median eminence projecting neuroendocrine neurons and “descending” preautonomic projecting neurons. At the rostral limit of the PVN, periventricular SS neuroendocrine parvocellular neurons are found and continue to be found near the ventricle for most of the nucleus. More caudally, THR and CRH neurons are interspersed with magnocellular neurons in both the medial magnocellular and posterior medial magnocellular compartments although at this level most CRH neurons are found at the lateral edge. Even more caudally, the parvocellular and magnocellular compartments tend to re-segregate as more of the parvocellular neurons are centrally projecting pre-autonomic regulators.

Generally, the dendrites of the PVN are simple and range from single dendrites on bipolar cells to two or three dendrite multipolar neurons with minimal additional dendrite branching (Swanson and Sawchenko, 1983). The majority of axons from neuroendocrine neurons of the PVN project to only one neuroendocrine secretory location (median eminence or posterior

pituitary) with some additional locally projecting axon collaterals. There are some neuroendocrine neurons that also project centrally to preganglionic autonomic centers. Of the parvocellular descending pathway neurons, a significant portion connect to both sympathetic and parasympathetic regulating centers (Swanson and Huypers, 1980).

Once thought to be a fairly simple site of information relay, the PVN has recently been shown to be a site of integration and possesses electrophysiological and anatomical qualities that allow for more complex signal processing. These include glial cell amino acid release (Oliet et al., 2007), dendritic release of peptides (Ludwig and Lend, 2006; Ludwig and Pittman, 2003) and glutamatergic interneurons (Ferguson et al., 2008). Interestingly, direct soma-soma contacts with gap junctions are present in both OT and AVP containing neurons allowing for direct electrical coupling synchronization and pulsatile release (Van de pol, 1982; Andrew et al., 1981; Hatton and Tweedle, 1980). These connections are strengthened with suckling or dehydration stimulus. Gap junction proteins have also been found in parvocellular CRH and SS neurons (Westbert et al., 2009). Axon collaterals have been described to connect with dendrites of both parvocellular and magnocellular neurons in the PVN allowing for local integration (Van den pol, 1982).

Major neuroendocrine systems regulated by the PVN:

In regulation of metabolism, TRH release at the median eminence causes thyrotrophs in the anterior pituitary to make and secrete thyroid stimulating hormone (TSH), which then, stimulates production of thyroxine (T4), in the thyroid. T4 is processed into triidothyronine (T3) by deiodinases. Thyroid hormone (T3) regulates metabolism and gene expression by binding nuclear receptors throughout the body (Nillni, 2010).

The main circulating endocrine regulator of growth is growth hormone (GH). GH is released by anterior pituitary in response to growth hormone releasing hormone (GHRH), which is produced by cells from multiple nuclei in the hypothalamus, including the PVN although to a lesser extent than other nuclei such as the arcuate nucleus. The wide distribution of GHRH containing neurons highlights its evolutionary importance and, like the distribution of gonadotropin releasing hormone (which is absolutely required for reproduction), protects the animal from certain removal from the gene pool with damage to or altered development of a single hypothalamic nucleus. Counterbalancing the stimulatory action of GHRH on GH release is the inhibitory action of SS. SS is released from parvocellular neurons at the median eminence in physiological states where resource allocation to growth is inappropriate, e.g. stress or malnutrition (Muller et al., 1999).

The maintenance of proper water balance and blood pressure in animals is crucial to survival. The PVN and supraoptic nucleus receive inputs from several circumventricular organs, located outside of the blood brain barrier and with the ability to sense blood osmolarity and inflammatory markers. The PVN and SON also receive inputs from blood pressure sensors such as the Vagus nerve. These inputs act via Angiotensin, glutamate, GABA and other transmitters to regulate the release of AVP at the posterior pituitary from magnocellular neurons in the PVN and SON. AVP travels through general circulation to cause increased water reabsorption in the kidney (Yoshida, 2007).

In the process of lactation, emotional and suckling stimuli are carried to magnocellular neurons in the PVN and SON. Thought to be mostly regulated by noradrenergic mechanisms, oxytocin is released at the posterior pituitary, which is then carried to the mammillary glands where it stimulates production of milk and contraction for milk ejection (Lincoln and Paisley, 1982).

The HPA axis:

In the neuroendocrine stress response, neurons located in the PVN secrete CRH in the portal capillaries at the median eminence. This causes the release of adrenocorticotropic hormone (ACTH) from the corticotrophs into general circulation where it is then carried to the adrenal gland. Here, ACTH causes the production and release of glucocorticoids (corticosterone in rodents and cortisol in humans). Acutely, the increased concentration of circulating glucocorticoids cause the mobilization of energy stores and allows a heightened state of awareness. This is the hypothalamic – pituitary – adrenal (HPA) axis. There is also evidence that parvocellular CRH neurons can co-express AVP and when AVP and CRH act on corticotrophs together, they cause more robust ACTH secretion (Rittmaster et al., 1987). Glucocorticoids normally exert negative feedback regulation at multiple levels of the HPA axis. If elevated concentrations of glucocorticoids persist for extended periods of time, serious detrimental effects occur, including metabolic, immunological and psychological (Jankord and Herman, 2008)

Threatening stimuli or stressors are detected by the primary sense centers in the cortex and this information is passed to the hypothalamus after processing in limbic regions (Ferguson, 2008). Limbic regions influencing PVN CRH release have been reviewed by Jankord and Herman (2008). Briefly, CRH neurons are directly innervated by neurons of the bed nucleus of the stria terminalis, dorsal medial hypothalamus, preoptic area, and the nucleus of the solitary tract. These directly innervating regions receive inputs form the ventral subiculum (glutamatergic), medial prefrontal cortex (glutamatergic), medial and central amygdala (GABAergic) which communicate with the basolateral amygdala. It is thought that much of the glucocorticoid mediated negative feedback on the hypothalamus is mediated by glucocorticoid and mineralocorticoid receptors in the ventral hippocampus (outflow via ventral subiculum).

The central amygdala is thought to mediate responses to systemic or physiological stressors such as inflammation, whereas the medial amygdala is thought to mediate responses to perceived threat (psychological stressors) as in the case of acoustic or light stressors or forced swim test. Impact on HPA axis function from the medial prefrontal cortex depends on location and is different in prelimbic and infralimbic associated areas. Information from the limbic system appears to have two synaptic levels of processing before HPA axis activity is regulated. Physiological and psychological stressor information is likely weighed against the influence of other homeostatic systems at these synapses to determine appropriate HPA axis activation. It is also likely that these synapses allow for the limbic system to exert non-HPA axis mediated effects on other homeostatic systems.

In addition to negative feedback mediation at the hippocampus, Glucocorticoid receptors are also present in the amygdala, PVN and BNST, and prefrontal cortex (de Kloet et al., 1998). One theory of depression ontogeny involves prolonged periods of stress prior to the onset of depression symptoms. In fact the majority of individuals diagnoses with major depressive disorder can identify a specific and prolonged stressful event or series of events prior to their pathology. Humans diagnosed with major depressive disorder have been found to have decreased hippocampal volume and depressive symptoms also coincides with damage in the hippocampus that occurs in Alzheimer's disease (Swaab et al., 2005). This is consistent with findings in animal models where elevated levels of glucocorticoids lead to cell death in the hippocampus (Sapolsky et al., 1985), a site where negative feedback on the HPA axis is mediated. Cell death has also been described in the amygdala and other limbic structures after glucocorticoid treatment in animals (Zuloaga et al., 2012).

PVN Development:

There are two main organizational schemes in brain development; layers, as seen in the cortex and cerebellum and nuclei as seen in the hypothalamus. Due to the inability to categorize cells solely based on distance from ventricle or layer, developmental studies of the hypothalamus and thus PVN are intrinsically more complex than those investigating development of areas like the cortex. As in any tissue, the processes of proliferation, growth migration, differentiation, and death mediate the development of the hypothalamus. Process extension (axon and dendrite) and circuit integration are additional crucial steps in neuronal development. During development, each of these steps impact the other and altered regulation of one process can lead profound effects in downstream processes. The microenvironment and genetic program of the progenitor pool directs gene expression which influences migration. The microenvironment through which a cell migrates further influences its genetic program and receptor expression, which influence its growth, differentiation, process extension and circuit integration (Dodd and Kolodkin, 2005).

The hypothalamus is derived from the ventral lobe and the inferior lobe of the diencephalic neuroepithelium (neural tube state). Altman and Bayer (1983, also available at http://neurodevelopment.org) describe three waves of neurogenesis in development of the rat hypothalamus. The first wave is between E13 and E15 (mouse E11 – E13) and these neurons form lateral hypothalamic structures. The second wave is between E15 and E17 with these neurons becoming parts of the medial hypothalamus. The final wave occurs between E17 and E19. These neurons make up the periventricular hypothalamic populations. The development of the paraventricular nucleus seems be an exception to the general rule of hypothalamic development although similar patterns remain. Specifically, though a periventricular and medial structure, magnocellular PVN neurons label with tritiated thymidine at E13 and E14

which is the same time that similar magnocellular neurons of the supraoptic nucleus, which is a lateral structure, are labeled. Within the PVN there is a lateral to medial gradient in time of neurogenesis and parvocellular neurons are labeled later in development than magnocellular neurons. This general pattern of earlier formed cells migrating farther than later formed neurons is opposite of the pattern seen in cortical development where the later formed neurons must migrate past older cells. There also appears to be a rostral to caudal gradient in neurogenesis as rostral cells are formed before caudal cells within the same medial to lateral level. Based on time of label and general pattern of label over time, sites of origin are also described, although one could argue that interpreting these general patterns of labeling at such long time intervals is inappropriate to determine sites of origin and may be possibly misleading. That said, Altman and Bayer worked with the most advanced techniques available at the time and put together a brilliant, disciplined, synthetic and seminal description of hypothalamic development that is still heavily relied upon. They suggest that the PVN neurons are derived from the neuroepithelium directly adjacent to the developing PVN.

The hypothalamus is induced to differentiate from the rostral diencephalon by the secretion of Sonic hedgehog (SHH) from the underlying prechordal plate. In mice lacking SHH no hypothalamic structures develop and SHH can induce hypothalamic development in cortical explants (Chiang et al., 1996, Ohkubo et al., 2002, Dale et al., 1997 and Ericson et al., 1995). After initial induction, SHH continues to be expressed in select hypothalamic progenitor pools and is necessary for glia differentiation as well as structure patterning (Alvarez-Bolado et al., 2012). Locally synthesized bone morphogenic protein (BMP) is also necessary to counter SHH signaling and contribute to structural patterning (Szarek et al., 2010).

Transcription factors involved in PVN development have been recently reviewed (Jo and Chua, 2009). In the development of the PVN, the orthopedia (OTP) transcription factor is

necessary for the generation of neurons that express OT, AVP, CRH, SS and TRH and any development of PVN, SON or Anterior periventricular nucleus (Acampora et al, 1999, Wang and Lufton, 2000). Downstream of OTP are the obligate dimerizing transcription factors SIM1 and ARNT2. Knockouts for either SIM1 or ARNT2 have similar phenotypes as OTP knockouts (Michaud et al., 2000 and Keith et al., 2001). Downstream of the SIM1/ARNT2 heterodimer are the transcription factors BRN2 and SIM2. BRN2 is necessary for the generation of neurons that express AVP, CRH and OXT (Nakai et al., 1995, Shonemann et al., 1995) while SIM2 is necessary for the generation of neurons that express SS and TRH in development. It is unclear how selective expression of BRN2 or SIM2 is achieved in the presence of the same SIM1/ARNT2 dimer. Survival and differentiation of neurons that express CRH, OT and AVP is also dependent on expression of PIT-1 at the pituitary and thought to be mediated by neurotrophin signals to the developing axons as they extend ventrally (Li et al., 1990, Godfrey et al., 1993, Lin et al., 1993 and Schonemann et al., 1995). SOX 3 is another transcription factor expressed in the developing PVN that has been linked to neuroendocrine disorders (Szarek et al., 2010). Genomic analysis combined with high throughput in situ hybridization has recently been accomplished to determine molecules that are present during hypothalamic development (Shimogora et al., 2010).

There are several factors that can be categorized as guidance cues, which are found in unique patterns in the developing hypothalamus suggestive of them influencing PVN development. One of the most striking patterns is that of GABA and the enzymes necessary for its production, glutamic acid decarboxylase (GAD) 65 and 67. The embryonic PVN is ringed by regions of densely immunoreactive GABA and GAD while there is an absence of GABA and GAD within the developing PVN. Interestingly, $GABA_A$ and $GABA_B$ receptor subunits are enriched in the developing PVN (McClellan et al., 2010). Independent support of this hypothesis was found studies of HES1. HES1 is a Notch effecter gene, which is expressed in

the developing pituitary and hypothalamus. In mice lacking HES1, there was altered distribution of GAD 67 immunoreactivity, which corresponded with altered placement of AVP immunoreactive neurons and fibers (Aujla et al., 2011).

Another unique pattern suggestive of a role for guidance cues is found in the distribution of the semaphorin receptors, Plexin A1 and Plexin C1. Plexin A1 is found in regions surrounding the PVN, while Plexin C1 is found within the PVN. In Plexin C1 mutant mice, OT and AVP neuron distribution was disrupted (Xu and Fan, 2007). Also, the dorsal and ventral boundaries of the PVN appear to be outlined by Slit1, Neuropilin 1 (co receptor for semaphorin) and semaphorin 3F (guidance protein) at E15, whereas Neuropilin 2 and Semaphorin 3C are found within the PVN (Xu and Fan, 2008). Some of these molecules might also be important for the segregation of specific compartments within the PVN.

The PVN is not discernable as a cell group using Nissl stains until E17. However, many of the neurons that make up the PVN are present earlier and express immunoreactive peptide before E17. For example, immunoreactive CRH and AVP can be found in the PVN by E15. Chapters 2 and 3 contain detailed descriptions of when key PVN components are detectible via immunohistochemistry. It is not yet known what event occurs between E15 and E17 to allow for Nissl stain detection, although possible mechanisms include proliferation of glia and/or tighter cell packing mediated by migration.

Neuron Migration:

Several human disorders have been described that are a direct result of altered neuron migration in the cortex. Type I and type II lissencephaly, neurodevelopmental disorders where brain sulci and gyri fail to form, are due to neurons either not making it to the cortical plate or migrating too far and over the marginal zone. Mutations in LIS1, 14-3-3ε, DCX, RELN and ARX can all lead to lissencephaly by causing altered neuron migration (Kanatani et al., 2005). Neuron migration can be described by three steps; first extension of the leading process, then translocation of the soma and nucleus and finally the retraction of the trailing process. For these processes to work the cell must attach to a substrate to allow for traction (Zheng and Poo, 2007). Transmembrane proteins that interact with extracellular matrix or proteins on the membranes of neighboring cells mediate this attachment. Many of these interactions are directed based on glycoproteins. When genes regulating the glycosylation of the extracellular matrix glycoprotein, α-dystroglycan, are mutated, migration deficits cause Walker-Warburg syndrome (Beltran-Valero de Bernabe et al., 2002) and muscle-eye-brain disorder (Yoshida et al., 2001).

At the site of adhesion, a slew of structural, cytoskeletal, and signaling molecules come together under the plasma membrane to form the focal adhesion complex (reviewed in Zheng and Poo, 2007). The focal adhesion complex attaches the cytoskeleton an anchoring point from which contraction forces can be exerted. These complexes must be rapidly assembled and dissembled to allow for migration. They are broken down prior to soma translocation and retraction of the trailing process. Process extension is initiated by assembly of actin cytoskeleton, which is followed by microtubule extension. Motor proteins work to pull the soma toward the leading process. Thus, there are multiple sites where drugs or signals can influence the direction and rate of migration. These include A) initial ligand receptor interaction

at the cell membrane, B) subsequent receptor activation and signaling to recruit actin cytoskeleton and actin modifying enzymes, C) targeting of microtubules to actin assemblies at the leading protrusion and enzymes mediating microtubule dynamics, D) motor protein activity and E) the assembly and disassembly of focal adhesion complexes.

Medial to lateral migration is aided by the presence of radial glia, which arise at the stage of the neural tube and stretch from the ventricle to the pia surface. This creates a series of railroad track like fibers that immature neurons can crawl along during the course of migration (Rakic 1972).

GABA and its Receptors:

$GABA_B$ receptors, among the first G protein coupled receptors to be identified, were cloned after searching for molecules that could explain responses to GABA that were insensitive to chloride and bicuculline. They have been extensively studied and excellent reviews are available (Gassmann and Bettler, 2012 and Froestl et al., 2001). The functional G protein coupled receptor is a heterodimer of R1 and R2 subunits. Ligand binding occurs at the R1 subunit and G protein interaction is mediated by the R2 subunit. Several splice variants in the R1 subunit have been identified. Interestingly, the R1a variant is expressed predominantly before birth, while the R1b variant is expressed predominantly after birth. The major difference between R1a and R1b is located in domains on the extracellular side. These domains are thought to mediate trafficking and pre vs. postsynaptic location via association with transmembrane protein in vesicles. More R1 variants exist but are less well characterized and their influence on GABA signaling is unknown. Embryonically, the R1 subunit is expressed at higher levels than the R2 subunit (Lujan et al., 2005). There is also some evidence for splice variants in the R2 subunit, although they are less well characterized.

$GABA_B$ receptor activation has been shown to affect the adenylate cyclase system and decrease Ca++ conductance and increase K+ conductance. Mostly thought to be mediated by pertussis toxin sensitive $G_i\alpha$ and $G_o\alpha$, some pertussis toxin insensitive baclofen effects have been found in magnocellular neurons of PVN and SON and in presynaptic receptors. Also there appears to be an age dependent ability of baclofen to increase GTP binding. These results indicate that regional and developmental regulation of G protein and receptor association exists (Bowery et al., 2002). Depending on the type of other stimulation present, $GABA_B$ signaling can enhance or diminish cAMP. Specifically, in the presence of no exogenous stimulation or forskolin stimulation, $GABA_B$ activity inhibits adenylate cyclases. In the presence of beta adrenergic stimulation, $GABA_B$ activity increases cAMP production (Bowery and Enna, 2000). More recently, a class of auxiliary subunits has been identified that bind to the B2 subunit and dramatically alter the pharmacology and kinetics of the $GABA_B$ receptor (Pinard et al., 2010).

$GABA_A$ receptors are a family of ligand gated Cl^- channels. Five subunits come together to make the functional channel. There are at lease 21 subunits that can be used to build the channel (Lujan et al., 2005). Depending on the concentration gradients of Cl^-, the activation of $GABA_A$ receptors can exert a depolarizing or hyperpolarizing influence on the membrane potential. In embryonic development, Cl^- concentrations are higher inside the cell than outside. This gradient reverses after birth with the expression of the K^+/Cl^- co transporter KCC2, which shunts Cl^- ions to the outside of the cell (Lu et al., 1999). There have been several reports of the embryonic gradient being present in specialized locations within the adult brain, for example the progenitor pool of the adult hippocampus (Liu et al., 2009) and some magnocellular neurons in the PVN, which coincide with a lack of KCC2 expression (Haam et al., 2012; Kim et al., 2011).

GABA has been shown to elicit multiple effects on neurodevelopment. These include effects on proliferation (Ben-Yaakov and Golan et al., 2003, Luyt et al., 2007), migration (Behar et al., 1996, 1998, 1999, 2000, 2001, Bolteus and Bordey, 2004, Cuzon et al., 2006, McClellan et al., 2008), DNA synthesis (Haydar et al., 2000), differentiation (Salazar et al., 2008), growth and morphology (Represa and Ben-Ari, 2005). It has been shown that these effects are through non synaptic, SNARE independent release of GABA during embryonic development (Manent et al., 2005). Thus the unique pattern of GABA/GAD immunoreactivity that surrounds the developing PVN (see PVN Development above) could regulate a number of processes necessary for proper formation of the PVN.

GABA and Anxiety and Depression:

For some time investigators have suspected a link between GABA and disorders related to HPA axis dysfunction (Nemeroff, 2003 and Cryan and Kaupmann, 2004). Anxiety and depression are the most common outcomes of stress in humans and animals (Kalueff and Nutt, 2007). Interestingly there is a high rate of comorbidity of anxiety and depression (Freeman et al., 2002, and Nutt, 2005). There are decreased GABA concentrations in the plasma (Petty et al 1998), CSF and brain (Sundman-Eriksson and Alard, 2002, Kugaya et al., 2003, Epperson et al., 2006), and fewer GABA neurons in the orbitofrontal cortex of individuals with depression. Using mice as a model animal these findings were repeated showing that mice could be induced to have anxiety-like and depression–like symptoms through environmental manipulation (stress, social isolation or learned helplessness (Kalueff and Nutt, 2007)). Also, there is a link in polymorphisms of the GAD 65 gene with anxiety behavior in children (Smoller et al., 2001). In model animals, disrupting the genes that encode GAD (Kash et al., 1999, and Stork et al., 2000), $GABA_A$ (Crestani et al., 1999), or $GABA_B$ (Mombereau et al., 2004 and Gassmann et al., 2004) receptors all cause anxiety-like phenotypes and some

also cause changes in depression-like behavior. Finally, when animals lack GABA transporter subtype 1, which pulls extracellular GABA back into the neuron, anxiety and depression-like behaviors are reduced (Liu et al., 2007).

There is ample evidence that GABA directly regulates both activity of the HPA axis at the PVN (Cullinan et al., 2008) and gene expression in the PVN (Bali and Kovacs 2003, and Gao and Bao et al., 2010). Few studies exist, however, investigating GABA's role on development of brain regions that regulate HPA axis and none attempt to tie GABA mediated development with HPA axis function and related disorders.

Sex Differences in HPA Axis function and stress related disorders:

Anxiety and depression disorders in general (exceptions do exist e.g. bipolar depression) affect more females than males (Viveros et al., 2012). Basal and stimulated HPA axis activity has been shown to be different between the sexes (Critchlow et al., 1963 Kant et. al., 1985, Handa et al., 1994, Iwasaki-Sekino et al., 2009). Generally, females show increased stress responses for a longer period of time than males. This corresponds to sex steroid influences on the expression of peptides in the PVN (Haas and George, 1988 and Patchev et al., 1995). These differences in HPA activity and peptide expression have been shown to be sensitive to hormone treatments neonatally (Patchev et al., 1999).

Sex steroids have also been shown to influence molecules that modulate the HPA axis. Signaling and trafficking of CRH receptors is different in males and females (Bangasser et al., 2010). Mecp2, a transcription factor involved in the expression of CRH and implicated in Rett Syndrome, is expressed differently in males and females (Kurian et al., 2007). The $GABA_B$ R1a subunit is significantly more abundant in hypothalamic block homogenates from females

that males at birth (Bianchi et al., 2005). Kelly and colleagues have shown the estradiol and some other sex steroid metabolites can attenuate $GABA_B$ receptor activation with baclofen through membrane estrogen receptors (Qiu et al., 2008). Finally, susceptibility to synthetic glucocorticoid induced apoptosis differs between males and females (Zuloaga et al., 2011, 2012).

Few have attempted to investigate specific processes in embryonic development to determine how the HPA axis is formed differently in males and females and how this might contribute to disease susceptibility. Those that have, typically use a model of maternal stress that shows a female protective affect (males more susceptible). This does not correlate with the pattern seen in humans and is of questionable utility in determining factors of development that can go awry and lead to disease susceptibility in the largest human populations. The aim of this work was to test hypotheses regarding GABA's action on PVN development and importantly, tie altered PVN development to HPA axis function and related disorders.

Chapter 2 illustrates how the hypothesis that GABA acts through the $GABA_A$ receptor to direct PVN development was tested. Through embryonic $GABA_A$ receptor blockade with the receptor antagonist bicuculline, it was found that female mice exposed to the antagonist embryonically had significantly less immunoreactive estrogen receptor (ER) α in and around the developing PVN. This chapter consists of excerpts from a manuscript for which Kristy McClellan was first author. Excerpts chosen to be included in this dissertation were those that I had significant and direct involvement in (McClellan, Stratton and Tobet, Journal of Comparative Neurology, 2010).

Chapter 3 examines $GABA_B$ receptor regulation of the peptide content of corticotropin releasing hormone, a peptide secreted by the PVN to initiate the neuroendocrine stress response. This work utilized mice without functional $GABA_B$ receptors and a special semi-quantitative

immunohistochemistry approach to show a sex difference that was region specific for the influence of $GABA_B$ receptors on CRH peptide levels in the PVN (Stratton, Searcy and Tobet. Physiology and Behavior, 2011).

The most significant work presented here is located in chapter 4. This chapter shows that $GABA_B$ receptor antagonism caused immature neurons in the developing PVN to increase their speed of movement. This increase in movement speed was accompanied by a lateral shift in the placement of neurons in the adult animal after embryonic $GABA_B$ receptor antagonism. Finally, this altered PVN cytoarchitecture corresponded with increased anxiety-like behaviors in females, hyperactivity in males and decreased depression-like behaviors in both sexes, again in adult mice that were embryonically exposed to $GABA_B$ receptor blockade. This embryonic $GABA_B$ receptor antagonism almost completely reproduced the phenotypes seen in the receptor knockout mice (Stratton et al., in preparation).

The dissertation ends with a discussion of how our findings enhance the understanding of PVN development and the sex dependent nature of HPA axis dysfunction. Included in this section is a list of open research questions and attractive experimental options to address these questions. The chapter ends with a brief summary of unpublished findings and abandoned projects.

CHAPTER 2. ROLES FOR GAMMA-AMINOBUTYRIC ACID IN THE DEVELOPMENT OF THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS

Summary

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. A number of molecular markers were noted within the developing PVN, including estrogen receptors (ER), neuronal nitric oxide synthase (nNOS) and brain derived neurotrophic factor (BDNF). There was also a notable enrichment of immunoreactive $GABA_B$ and $GABA_A$ receptor subunits in the PVN. By contrast, immunoreactive GABA was found in cells and fibers surrounding the PVN with little or no immunoreactivity found within the PVN. Treatment with bicuculline to decrease $GABA_A$ receptor signaling from embryonic day (E)10 to 17 resulted in fewer cells containing immunoreactive (ir)-ERα in the region of the PVN versus control. Based on the current study, the PVN can be added as another site where GABA exerts morphogenetic actions in development.

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 in cells 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 different cell phenotypes that delineate regions within the PVN are expressed early in development. Estrogen and glucocorticoid receptors are among those found at embryonic ages (Owen and Matthews, 2003; Tobe et al., 2005). Neuroactive peptides such as OT and AVP (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., 1998; Xu and Fan, 2007). These cells are derived from precursors along the third ventricle with distinct timelines; more lateral (magnocellular) cells are the first to undergo their final mitotic division and move away from the third ventricle, between E10.5 and E12.5, in the developing mouse (Karim and Sloper, 1980; Okamura et al., 1983). Cells that occupy the more medial (parvocellular) region of the PVN are generated later.

Differentiation of the PVN requires the expression of particular transcription factors, including Sim1 (Michaud et al., 1998), 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 suggesting that Sim1 is required for terminal cell differentiation of these cell types in both the PVN and SON

(Michaud et al., 1998). More recently it has been suggested that Sim1 expression may also be important for 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 showed 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).

Migration at the cellular level is mediated by molecular communication between cells often from small molecules or polypeptides released to diffuse through extracellular space. 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). GABA has been shown to influence cell movements within one hypothalamic nucleus, the ventromedial nucleus (VMN), through $GABA_A$ (Dellovade et al., 2001) and $GABA_B$ (Davis et al., 2002; 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 to the developing PVN, which they surround to determine if similar roles might be in play.

To determine the potential role of GABA acting through $GABA_A$ receptors, pregnant mice were administered bicuculline injections to examine cell positions and immunoreactive ERα in the PVN region of fetal brains. 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). The location of immunoreactive ERα cells has previously been shown to be a useful marker for

identifying alterations in cell position in other regions of the developing hypothalamus (Tobet et al., 2002).

Materials and methods

Animals:

Animals were mated overnight and checked for vaginal plugs the following morning. The day of plug was designated as E0. 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 embryos were removed individually before being perfused with 2mL (E15 and E17) or 5mL (P0) 4% paraformaldehyde in 0.1M phosphate buffer using a hand held 10ml syringe. Body weight and/ or crown rump length measurements were taken to verify ages (E15, CRL= 14-15mm; E17, BW= 0.7-0.95g, CRL= 17-18mm). Sex determination was made through direct inspection of the gonads or PCR analysis for the Y-chromosome sry gene. Brains were post-fixed in 4% paraformaldehyde overnight and were placed in 0.1M phosphate buffer and stored at 4°C until tissue sectioning. To generate embryos with decreased GABAA receptor activation during the time of PVN development, pregnant dams were injected (s.c.) with 1mg/kg (Bless et al., 2000; Nguyen-Ba-Charvet et al., 2004) bicuculline methobromide (B-4013, Sigma, St. Louis, MO) in 100µl water twice per day from the evening of E10 thru the morning of E17 (only one injection on E10 and E17). Vehicle treatment was with 100µl water alone. Embryos were transcardially perfusion fixed as described above on the afternoon of E17. 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:

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

Nissl staining:

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). Selected sections were processed for immunohistochemistry and counterstained following 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.

Immunohistochemistry:

Brain tissue collected from pups at ages E15, E17, and P0 were embedded in 5% agarose and cut into 50µm or 60µm thick coronal sections using a vibrating microtome (Leica VT1000S). Alternating sections were collected in 0.05M phosphate buffered saline (PBS), pH 7.5. Excess unreacted aldehyde 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 (Table 1) were: ERα (1:5000; C1355, Upstate Biotechnology, Charlottesville,

VA), BDNF (1:200; sc-546, N-20, Santa Cruz Biotechnology, Santa Cruz, CA), CRH (1:25,000; generously provided by Dr. W. Vale), vasopressin (1:10,000; Immunostar, Hudson, WI), ERβ (1:500; early lot number 10967002 of Z8P, Zymed Laboratories, San Francisco, CA), nNOS (1:10,000; Immunostar), calbindin (1:5000; AB1778, Millipore, Billerica, MA and 1:40,000; D-28K, Sigma-Aldrich, St. Louis, MO), galanin (1:8000; Millipore (Brown et al., 1999), GABA (1:500; Immunostar), GAD67 (1:5000; Chemicon), NPY (1:8000; Immunostar), $GABA_A$ receptor subunits γ2 (1µg/ml; generously provided by Dr. W. Sieghart) and α1 (1:1000; Phosphosolutions, Inc.). 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% diaminobenzidine with 0.02% nickel and 0.02%H2O2 diluted in tris-buffered saline (pH 7.5). Pre-incubation with peptide served as an antibody control for BDNF (5µg peptide: 1µg antiserum for 1 hour at room temperature, data not shown), while similarity of immunoreactivity among multiple antisera served for others (e.g., calbindin, nNOS, and comparison of GABA to GAD; see below and Table 1), similarity to literature reports for others (e.g., AVP, OT, galanin), as well as omission of primary antisera that resulted in non-detectable reaction product.

TABLE 1.

List of Antibodies Used for Tissue Analysis and Those Used To Verify Specificity

Primary antibody	Catalog No.	Lot No.	Species	Immunogen	Company
ERα (C1355)	06-935	JBC1371375	Rabbit	TYYIPPEAEGFPNTI	Millipore
BDNF (N-20)	sc546	A0207	Rabbit	Human BDNF aa128–147 RHSDPARR-GELSVCDSISEW GI-P23560	Santa Cruz Biotechnology
ERβ (Z8P)	51-7900	10967002	Rabbit	Mouse ERβ aa 468–485 CSTEDSKSKEGSQNLQSQ	Zymed Laboratories
CRH	N/A	N/A	Rabbit	Rat hypothalamic CRH	Provided by Dr. Wylie Vale
Vasopressin	AB1565	17090354	Rabbit	Whole arginine vasopressin conjugated to thyroglobulin	Millipore
nNOS	24287	436002	Rabbit	Human C-terminal peptide aa 1419–1433	Immunostar
Calbindin	AB1778	LV1378360	Rabbit	Recombinant mouse calbindin	Millipore
Calbindin	C9848	117K4757	Mouse monoclonal	Bovine kidney calbindin-D	Sigma-Aldrich
GABA	20094	517211	Rabbit	GABA coupled to BSA	Immunostar
GAD67	AB5992	unknown	Rabbit	Recombinant feline GAD67	Chemicon
$GABA_BR1$ (GP311)	N/A	N/A	Guinea pig	RQQLRSRRHPPTPPDPSGGLPRGPSE	Provided by Dr. Margeta-Mitrovic
NPY	22940	550212	Rabbit	Synthetic porcine NPY conjugated to BSA	Immunostar
Galanin	AB1985	18010442	Rabbit	Human galanin	Millipore

Antibody Characterization:

Specific information on company, lot number and immunogen can be found in table 1. Below is additional evidence of antigen specificity for antisera and antibodies used in this paper produced in our lab or taken from the literature.

ERα (C1355)

This antiserum recognizes a 66kDa band on Western blot using in vitro translated ERα, rat pituitary and uterine lysates as well as lysates from COS-1 cells transfected with rat ERα but not in untransfected COS-1 cells and does not cross react with ERβ (data provided by supplier; Millipore; Friend et al. 1997). In addition, others have shown that preadsorption with ERα peptide eliminated immunoreactivity while preadsorption with ERβ did not change immunoreactivity in rat CNS (Papka et al., 2001).

BDNF (N-20)

The pattern of immunoreactivity seen with this antiserum closely matches the pattern of reactivity seen by in situ hybridization studies with a riboprobe recognizing BDNF mRNA. In addition, when preadsorbed overnight at 4°C with 5 µg of blocking peptide provided by Santa Cruz (sc-546 P) in 1 ml primary antibody buffer with 1 µg BDNF (N-20) antiserum, immunoreactivity was eliminated.

ERβ (Z8P; lot number 10967002)

Others have shown that this antiserum recognizes in vitro translated rat ERβ but not ERα and an ERβ-like 60-kDa protein from rat granulosa cells and ovary extracts on Western blots, and that the immunoreactivity pattern of this antiserum overlaps the mRNA pattern of ERβ by in situ hybridization (multiple regions in rat CNS) (Shughrue and Merchenthaler, 2001). We have further determined that immunoreactivity was absent in formaldehyde fixed brain tissue from ERβ knockout mice (generously provided by P. Bonthuis and Dr. E.F. Rissman, University of Virginia School of Medicine).

CRH

On dot blot there was no cross-reactivity with the peptides, melanin concentrating hormone or αMSH while CRH peptide was strongly recognized (Van Bockstaele et al., 1996). In addition others have shown that immunolabeling in rat was eliminated by preadsorption with synthetic CRH (Sigma C-3042) (Tagliaferro et al., 2008). The pattern of CRH immunoreactivity in the PVN for the current experiments is consistent with prior results in mice (e.g., Keegan et al., 1994; Alon et al., 2009).

Vasopressin

The manufacturer has shown that this antiserum does not cross react with oxytocin peptide in western blots (data provided by supplier; Millipore). Previous studies have also shown that preadsorption with synthetic vasopressin peptide (10μM) resulted in a complete loss of immunolabeling (Das et al., 2007). The pattern of vasopressin immunoreactivity in the PVN for the current experiments is consistent with prior results in mice and rats (e.g., Vacher et al., 2002; Somponpum and Sladek, 2003).

nNOS

We have found no specific immunoreactivity using a C-terminal directed antiserum (Immunostar 24287) in nNOS knockout mice (exon 6 deletion generously provided by P. Huang, Massachusetts General Hospital and Harvard Medical School; Gyurko et al., 2002). Use of an N-terminal specific nNOS antiserum (Zymed, cat 61-7000), produced a similar pattern of immunoreactivity in wild type mice and also failed to detect immunoreactive protein in the knockout (as also reported in Gyurko et al., 2002).

Calbindin

Two calbindin immunoreagents have been used, one rabbit polyclonal antiserum (AB1778; Millipore) and one mouse monoclonal antibody (C9848; Sigma-Aldrich). The pattern of immunoreactivity was similar between the two immunoreagents and matched that seen in our previous studies of embryonic mouse hypothalamus (Edelmann et al., 2007). AB1778 has a specific reaction product of 28kD on Western blots as stated by the manufacturer and seen in our own studies (adult mouse hypothalamic homogenate). Others have shown a lack of immunoreactivity in mouse (C9848) following preadsorption with 100μM calbindin peptide (Huynh et al., 2000).

GABA

The specificity of the antiserum was evaluated using competitive inhibition enzyme-linked immunosorbent assay. Preadsorption of this antiserum with conjugates of GABA completely eliminate labeling, while preadsoprtion with other conjugates (glutamate, aspartate, beta alanine, tyrosine, taurine, glycine, alanine) did not inhibit the antiserum's ability to bind GABA (data provided by supplier; Immunostar). There is notable similarity of immunoreactivity for GABA and its synthetic enzyme GAD67 in fetal mouse hypothalamus (Tobet et al., 1999).

GAD67

This antiserum preferentially recognized GAD67 over GAD65 on Western blots of recombinant proteins and rat brain homogenate illustrated by a band at 67kDa (data provided by supplier; Chemicon). The immunoreactivity pattern was similar to mRNA by in situ hybridization (multiple regions in rat CNS) and preadsorption with bacterially produced rat GAD65 did not change the immunoreactivity pattern in rat brain while preadsorption with bacterially produced rat GAD67 blocked all specific immunoreactivity (Esclapez et al., 1994).

$GABA_BR1$

Others have verified specific immunoreactivity by preabsorbtion with an excess of immunogen peptide (Table 1) that led to a complete loss of immunoreactivity (Margeta-Mitrovic et al., 1999; Belenky et al., 2008).

NPY

Immunoreactivity was blocked with preabsorbtion of excess NPY but not with peptide YY, avian pancreatic polypeptide, b-endorphin, vasoactive intestinal peptide, cholecystokinin, or somatostatin (data provided by supplier; Immunostar). The pattern of NPY immunoreactivity for

the current experiments was consistent with prior results in the arcuate and PVN in mice and rats (e.g., Tobet et al., 1999; Dellovade et al., 2000; Legradi and Lechan, 1998).

Galanin

Preadsorption with excess porcine-galanin (H-6580, Bachem) resulted in loss of specific immunoreactivity in the region of the PVN in chick brains (Klein et al., 2006). The pattern of galanin immunoreactivity for the current experiments was consistent with prior results in the dorsal hypothalamic area in mice and rats (e.g., Brown et al., 1999; Wittman et al., 2004).

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). Four tissue sections from each embryonic brain contained a part of the PVN that was subdivided from rostral to caudal (Fig. 2.1; A-D). The sections were categorized based on the rostral / caudal location and angle of cut. Only those sections containing ERα immunoreactivity in a cell grouping lateral to the PVN and within the PVN were included in the analysis (Fig. 2.1; B). For the analysis of Bicuculline treated animals, cell counts were taken for sections of the PVN that contained ERα immunoreactivity in the region just lateral to the PVN. Cells containing ir-ERα were counted and designated as being within the region of the PVN or part of the cell group located just lateral to the PVN. The number of cells inside and outside the PVN was totaled for the left and right sides of the brain and averaged for each section. To account for possible over estimation of cell numbers, Abercrombie's formula was used to correct all cell counts, T/(T+h) where T=section thickness and h=mean object diameter (ir-ERα cell nucleus) (Guillery 2002). For E17 sections (Bicuculline vs Vehicle), the formula was 50µm/(50µm+5.6µm) resulting in a correction factor of 0.9.

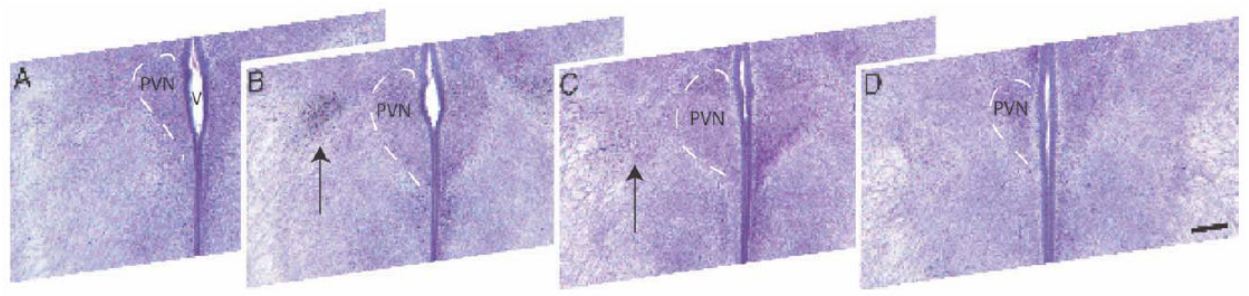

Figure 2.1. 50µm thick coronal sections taken tin the region of the PVN at E17. Sections contain IR ERα and were counterstained for Nissl substance to delineate the boundries of the PVN. This shows the rostral (A) to caudal (D) extent of the PVN. V, third ventricle, scale bar = 100 µm

For ir-ERα, statistical significance was determined by ANOVA for treatment (Bicuculline vs vehicle) × location (inside or outside the PVN as a repeated measure) using SPSS software (SPSS Inc., Chicago, IL).

Digital images for figures were acquired as noted above for analysis. Image adjustments were made using Adobe Photoshop software (version CS for Macintosh). Images in figure 1 were converted to a composite figure using Adobe Illustrator (version CS for Macintosh). All other images were converted to grayscale and sizes were adjusted for the appropriate resolution to create the final composite figures. Images were enhanced for contrast by adjusting levels and using the unsharp mask tool to improve clarity.

Results

PVN cell phenotypes at E15:

Several immunohistochemical characteristics selectively delineated 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 2.2 shows the distribution of a subset of immunoreactive markers that have differentiated by E15 in the PVN. These include cells containing CRH (Fig. 2.2A), vasopressin (2B), calbindin (2C), nNOS (2D), ERβ (2E), Erα (2F), galanin (2G), and NPY (2H). Based on the distribution of these various cell types it is clear that aspects of the cellular architecture are established at young ages. However, the size of the nucleus and the number of cells occupying the PVN increases with further development. Subnuclear compartments at the early ages would be difficult to assign. Of the molecular markers examined, immunoreactive NPY was the only one not intrinsic to cells of the PVN.

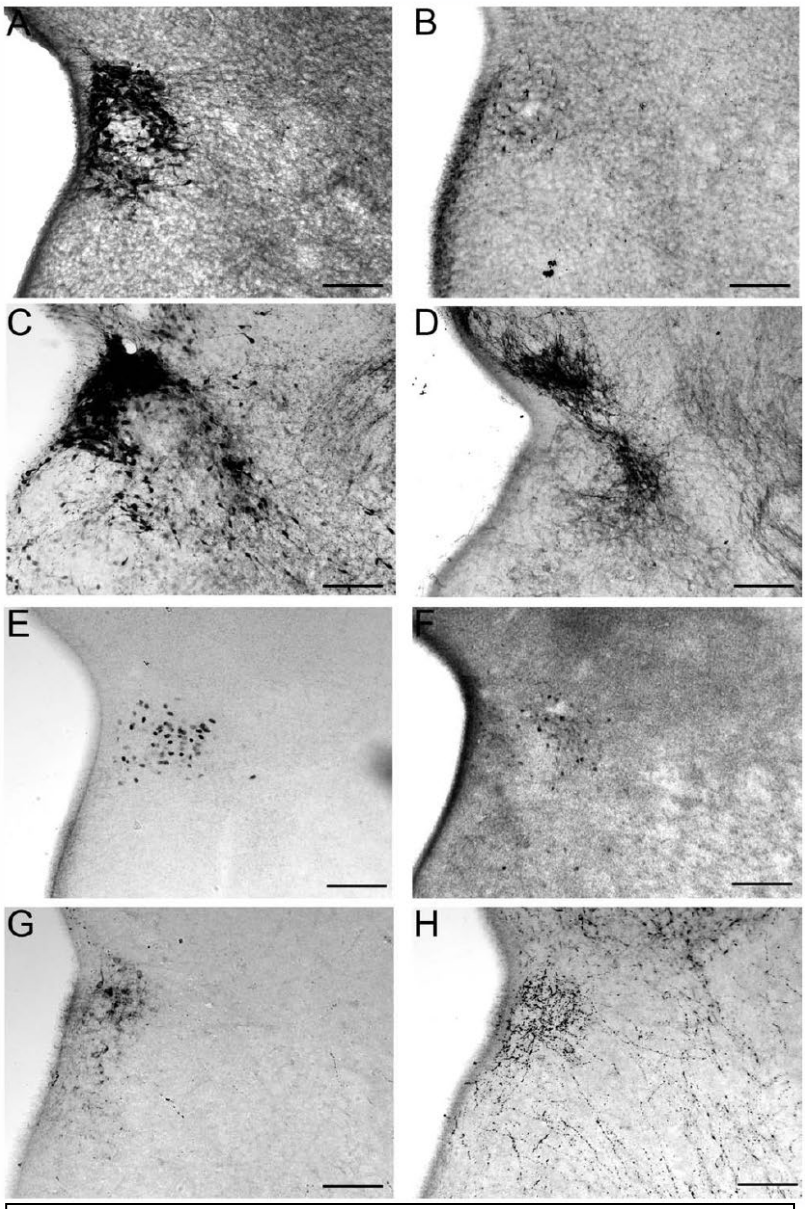

Figure 2.2. Several IR peptides, receptors and signaling molecules that populate the PVN by E15. CRH (A), AVP (B), Calbindin (C), nNOS (D), ERβ (E), ERα (F), galanin (G), NPY (H) delineated distinct populations of cells in the PVN at early ages. Scale bars = 50μm.

Unfortunately, cell bodies of origin were not discernible. 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 relatively unique to two regions within the developing hypothalamus - the PVN and the VMN. The progression from E13 (Fig. 2.3A), to E15 (Fig. 2.3B), to E17 (Fig. 2.3C), shows the dense populations of GABAergic cells and fibers that surround the PVN. By adulthood, GABAergic fibers completely fill the nucleus (data not shown).

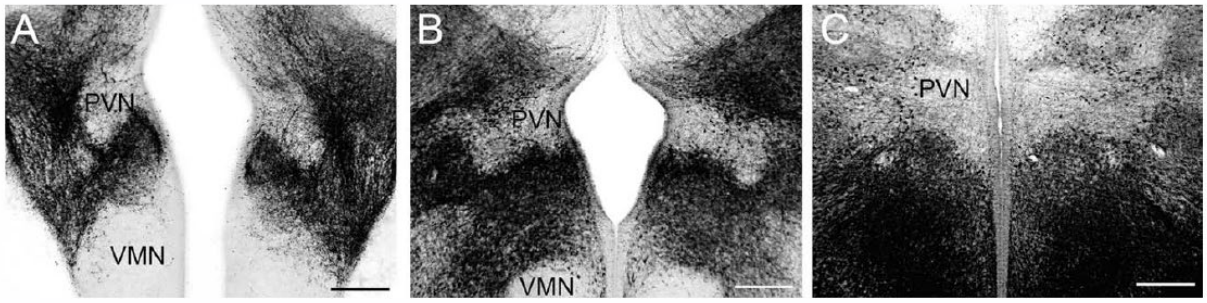

Figure 2.3. IR GAD 67 (A) or GABA (B,C) surround the region of the developing PVN. At E13 (A), E15(B), and E17(C) in the mouse, the PVN region is one of two hypothalamic regions where GABA is found surrounding the nucleus in development.The ventromedial nucleus of the hypothalamus (VMN) is the other nuclear group where this has been found. Scale bars = 100µm.

GABA signaling through the $GABA_A$ receptor influences the number of ir-ERα cells:

Pregnant mice were given daily bicuculline injections starting at E10 and embryos were perfused on E17. Sections taken for analysis correspond to those in Fig. 2.1B and 2.1C and are the only two sections that contain the lateral population of ir-ERα cells. Cell counts were taken for ir-ERα within the PVN and outside of the PVN on each side of the brain across two sections. Cell counts were combined from both sides of the brain and sections were analyzed as a two-way ANOVA for treatment by location as a repeated measure. There was a significant interaction between subjects in location by treatment ($F(1,4) = 50.1$, $p < 0.01$). Bicuculline treated pups had a 30% decrease in the total number of ir-ERα cells compared to the control (Fig. 2.4).

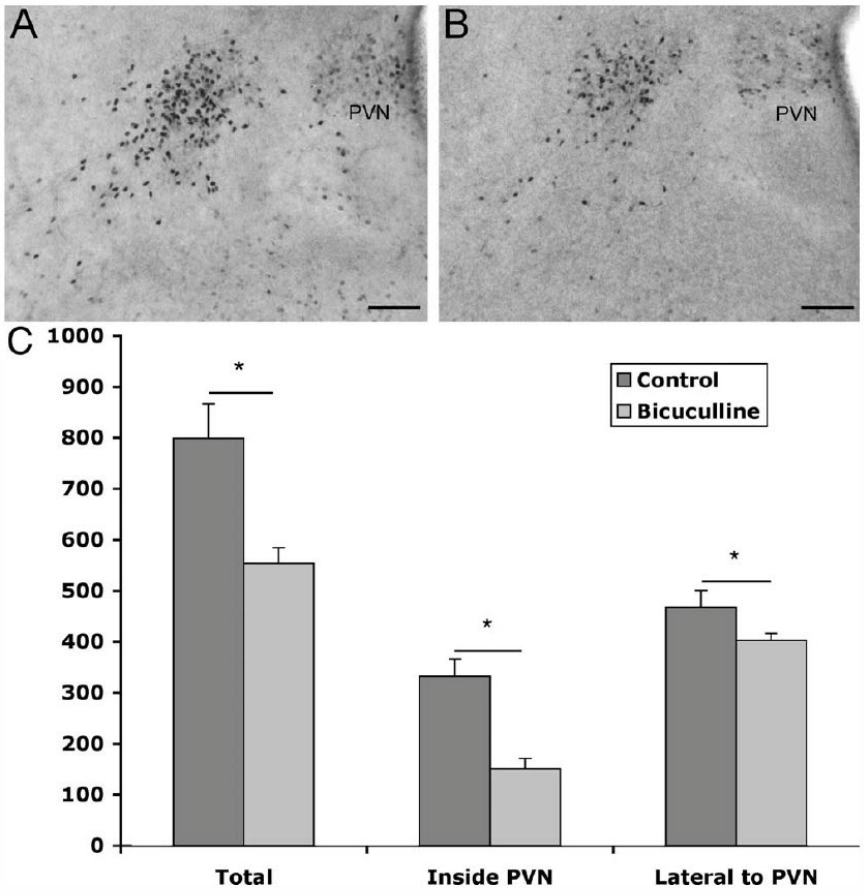

Figure 2.4. ERα IR at E17 in female mice exposed to bicuculline between days E10 and E17. Bicuculline treatment (B, n=3) compated with saline controls (A, n=3) caused about a 30% decrease in total ERα IR (C). IR ERα levels were lower both within the PVN and outside it following 1 mg/kg bicuculline treatment. There was a significant interaction between sumhects in location by treatment (*, $p<0.01$). Scale bars = 100μm.

While the decrease in ir-ERα cells was evident in cells just lateral to the PVN, there was a greater effect (more than a 54% decrease with bicuculline treatment) seen within the PVN.

$GABA_B$ and $GABA_A$ receptor subunits are found within the PVN at embryonic ages:

Adjacent sections were processed for ir-ERα and ir-$GABA_B$R1 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. 2.5A, B). $GABA_A$ receptor subunits are also located in cells within or around the developing PVN. Immunoreactive $GABA_A$ receptor subunit γ2 is notably found within the PVN (Fig. 2.5C) and immunoreactive α1 subunits (Fig. 2.5D) are found toward the periphery of the nucleus and in lateral locations.

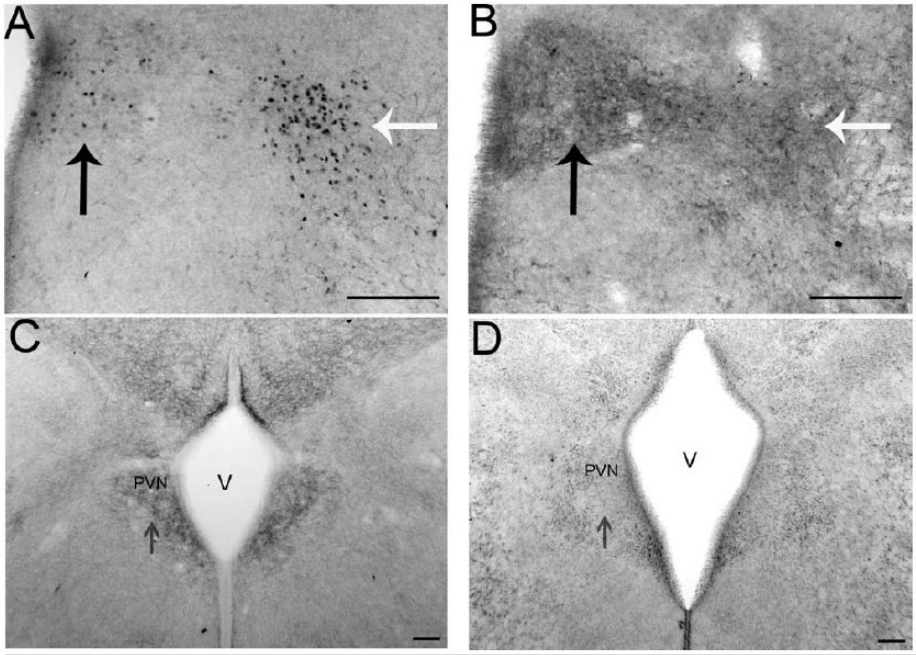

Figure 2.5. IR GABA receptor subunits and their relationship to ERα in the PVN. Adjavent sections with ERα (A) and $GABA_B$ R1subunit (B) at E17. Black arrows indicate IR within the PVN. White arrows point to IR lateral to the boundries of the PVN. Based on IR locations in adjacent section, these proteins may be expressed in some of the same cells. γ2(C) and α1(D) are two $GABA_A$ receptor subunits expressed in the PVN at E17. V, third ventricle. Scale bars = 100μm.

Discussion

Development of the hypothalamus involves the interactions 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. We have identified several molecules expressed early in PVN development; specifically BDNF, calbindin, nNOS, ERα and β, galanin and NPY. These markers and others (Caqueret et al., 2006), illustrate that a partially organized and heterogeneous nucleus is apparent before the PVN is discernible by Nissl stain. They provide tools to track the development of the PVN and identify molecules (GABA, NO, and BDNF) that could regulate the development of the PVN.

The current study delineated a ring like pattern of GABA and GAD expression in elements surrounding the PVN during embryonic development while $GABA_A$ and $GABA_B$ receptor subunits are enriched in cells of the developing PVN. This pattern is fundamental to the hypothesis that GABA acts to help form the boundary of the developing PVN and could provide gradient information to cells in the region. This GABA pattern of immunoreactivity is similar to one seen in the developing VMN where GABA effects on cell positioning were previously described (Dellovade et al., 2001; Davis et al., 2002; McClellan et al., 2006; McClellan et al., 2008). Before the establishment of axonal connections, GABA is not likely acting in its traditional role as a neurotransmitter at the level of the synapse (Taylor et al., 1990; van den Pol, 1997). The functional significance of GABA in early development may be to influence several developmental processes including proliferation, migration and differentiation (Lauder, 1993; Nguyen et al., 2001).

The original focus of this study was to identify GABA influences on cell position within the region of the PVN during a developmental time period when the PVN is being established as a

nucleus. Measurements of immunoreactive area do not represent total levels of protein. Additional studies would be needed to determine whether there are changes in the levels of protein content. The goal of this study was to determine where the cells containing specific proteins were distributed across the region. However, in our analysis we found evidence for potential differences in protein expression levels based on cell counts (ERα).

When $GABA_A$ signaling was disrupted, female mice exhibited an overall decrease in the number of cells containing ir-ERα, with a greater effect being found within the boundaries of the PVN. The overall decrease in the number of cells containing ir-ERα upon the disruption of $GABA_A$ signaling may be indicative of a decrease in the number of cells containing ir-ERα or the amount of protein being made in some of these cells rendering them undetectable. The difference in immunoreactive cell numbers between bicuculline and control treated mice could be mediated by GABA at several levels, ranging from gene expression to cell death. As the original focus of the current study was to examine influences on cell position, we did not conduct additional studies on the nature of the influence of $GABA_A$ signaling on cells containing ir-ERα..

$GABA_A$ and $GABA_B$ receptors work through two very different signaling pathways (ionotropic versus metabotropic) and this may explain the different roles they play in the development of the PVN. Both receptor types have been shown to play a role in VMN (Davis et al., 2002; Dellovade et al., 2000; McClellan et al., 2008) as well as cortical development (Behar et al., 1996; Behar et al., 1998). With regards to VMN development, both receptor types seem to play similar roles in migration, influencing the position and spread of ir-ERα cells as well as influencing the speed of migrating neurons. In the cortex, however, $GABA_A$ and $GABA_B$ receptors play different roles, influencing the likelihood of motion and the movement through individual layers (Behar et al., 1998). This study implicates a role for GABA in the differentiation (through $GABA_A$) of neurons immunoreactive for ERα in the PVN of female mice.

The lateral population of ERα cells that was altered in female mice with disrupted GABA signaling 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). Estradiol has been shown to enhance the sensitivity of PVN CRH neurons involved in the HPA response (Lund et al., 2006). Studies involving rats that were implanted with capsules releasing estradiol benzoate directly in the region of the PVN exhibited changes in their stress response (Lund et al., 2006). Although this response is largely thought to act through ERβ positive cells within the rat PVN, this response could be mediated through the lateral population of cells containing ERα of which about 70% are GABAergic neurons with projections to the PVN in the adult rat. In fact it has been shown that estradiol can block glucocorticoid dependent negative feedback on the PVN via ERα (Weiser and Handa, 2009). This lateral population of ERα containing cells may be part of the limbic inputs that exert an inhibitory tone on the PVN (Herman et al., 2005). Thus altering levels of ERα could change the ability of hormones to modulate the HPA axis. Hormone modulation of the HPA axis is not limited to estrogens as there is also evidence for androgens modulating HPA axis function at the level of the PVN (Williamson et al., 2005).

In the context of the current results, we reexamined the pattern of ERα immunoreactivity in the archived slides of 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 it is unlikely that the β3 subunit could be involved in the $GABA_A$ receptor response found in this study. With a

number of possible subunits in the developing PVN (e.g., Fig. 2.7) it is likely that other subunits are making up the $GABA_A$ receptors within the PVN (Fenelon et al., 1995).

The PVN is the ultimate regulator in 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 (Bao et al., 2008; Bao and Swaab, 2007; Wang et al., 2008). 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 impacts neurons developing in or moving through the region of the PVN. There is also evidence in the literature for changes in depression and anxiety with altered $GABA_A$ receptor signaling (Kalueff and Nutt, 2007).

GABA may potentially mediate aspects of PVN development to include ERα expression. GABA and ERα are important for regulating adult PVN and therefore HPA axis function. We suggest that some of the predisposition to affective disorders seen in humans and animal models with altered GABAergic signaling is due to altered formation of the PVN, a master regulator of the HPA axis.

CHAPTER 3. GABA REGULATES CORTICOTROPIN RELEASING HORMONE LEVELS IN THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS IN NEWBORN MICE

Summary

The paraventricular nucleus of the hypothalamus (PVN) is a major regulator of stress responses via release of corticotropin releasing hormone (CRH) to the pituitary gland. Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis is characteristic of individuals with major depressive disorder (MDD). Postmortem data from individuals diagnosed with MDD show increased levels of CRH mRNA and CRH immunoreactive neurons in the PVN. In the current study, an immunohistochemical (IHC) analysis revealed increased levels of CRH in the PVN of newborn mice lacking functional $GABA_B$ receptors. There was no difference in the total number of CRH immunoreactive cells. By contrast, there was a significant increase in the amount of CRH immunoreactivity per cell. Interestingly, this increase in CRH levels in the $GABA_B$ receptor R1 subunit knockout was limited to the rostral PVN. While GABAergic regulation of the HPA axis has been previously reported in adult animals, this study provides evidence of region-specific GABA modulation of immunoreactive CRH in newborns.

Introduction

The hypothalamic pituitary adrenal (HPA) axis is a major neuroendocrine component of physiological stress responses. Upon perception of a threatening or stressful environment, neurons in the paraventricular nucleus of the hypothalamus (PVN) release the peptide hormone corticotropin-releasing hormone (CRH). CRH then acts on cells in the anterior pituitary to cause the release of adrenocorticotropic hormone (ACTH), which stimulates the production and release of glucocorticoids from the adrenal gland (reviewed in Swanson and Sawckenko, 1983

and Herman et al., 2005). An acutely elevated level of cortisol is beneficial due to the mobilization of energy stores and the creation of a heightened state of alertness. However, prolonged periods of increased HPA axis activity are detrimental to the organism and associated with major depressive disorder and anxiety related disorders in humans (Bao et al., 2008). Both anxiety (Lewinsohn et al., 1998; Piggot, 1999) and depression (Noel-Hoeksema, 1997; Angst et al., 2002) are more prevalent in females than males. As the PVN is pivotal for HPA axis function and the integrator of threat, hormone, cognitive, and emotional information (Ferguson et al., 2008) the regulation of its output is critical. Based on post mortem studies, humans with depression have more CRH immunoreactive neurons in the PVN and increased levels of CRH mRNA (Raadsheer et al., 1994; Raadsheer et al., 1995).

Gamma aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the adult brain. There is mounting evidence to implicate GABA in anxiety and depression related disorders via signaling through $GABA_A$ and $GABA_B$ receptors (Kalueff and Nutt, 2007; Cryan and Kaupmann, 2005). $GABA_A$ receptors are pentameric ligand gated chloride channels and the targets of anxiolytic drugs like the benzodiazepines. $GABA_B$ receptors are heterodimeric G protein coupled receptors that are rendered non-functional by removal of either subunit (Prosser et al., 2001) and are also the targets of drugs thought to have anxiolytic activity (e.g., baclofen; Frankowska et al., 2007). The current study took advantage of mice with a genetic disruption of the R1 subunit of the $GABA_B$ receptor that eliminates functional activity. Previous studies suggested that decreased $GABA_B$ receptor signaling during development altered the cytoarchitecture of the hypothalamus including the PVN (McClellan et al., 2008; McClellan et al., 2010). In mice lacking functional $GABA_B$ receptors there were significant alterations in the locations of cells in or around the PVN containing immunoreactive estrogen receptor α and neuronal nitric oxide synthase (nNOS) fibers, as well as decreased levels of brain-derived neurotrophic factor (BDNF) immunoreactivity (McClellan et al., 2010). In the course of

investigating the role of $GABA_B$ receptor signaling in the placement of CRH neurons in the developing PVN there was a notable increase in the strength of CRH immunoreactivity in female $GABA_B$ R1 subunit knockout mice compared to wild type littermates. The current study, using a semi-quantitative immunohistochemical approach, was conducted to directly test whether $GABA_B$ receptor signaling regulates the levels of CRH immunoreactivity in the developing female PVN.

Materials and methods

Animals:

This study used a transgenic line of mice lacking functional $GABA_B$ receptors (McClellan et al., 2008, McClellan et al., 2010). 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). Heterozygous breeding pairs were used to generate homozygous null, heterozygous, and wild-type animals to be used in immunohistochemical studies. Animals were mated overnight and checked for vaginal plugs the following morning. The day that plugs were found was designated as embryonic day (E) 0. Pups were transcardially perfused on E13, E15, or postnatal day (P) 0. Pregnant mice were anesthetized with ketamine (80 mg/kg) and xylazine (8 mg/kg), and embryos were removed individually before perfusion with 2 ml (E13 and E15) or 5 ml (P0) 4% paraformaldehyde in 0.1 M phosphate buffer using a hand-held 10-ml syringe. Ages were verified by measurement of crown-rump lengths. Sex determination was made through direct inspection of the gonads and PCR analysis for the sry gene on the Y chromosome. Brains were postfixed in 4% paraformaldehyde overnight and then placed in 0.1 M phosphate buffer and stored at 4 °C until tissue sectioning. 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:

Genotyping of tail DNA was done as described previously (McClellan et al., 2008). Mice were genotyped for the $GABA_B$ R1 knockout allele and the sry gene using a standard Taq polymerase PCR kit (Qiagen, Valencia, CA). Results from pups heterozygous for the $GABA_B$ R1 knockout allele were pooled with wild-type mice as controls, since no heterozygote phenotypes have been observed for any characteristic in these mice.

Immunohistochemistry:

Brain tissue collected from pups at ages E13, E15, and P0 was embedded in 5% agarose and cut into 50 µm thick coronal sections using a vibrating microtome (Leica VT1000S). Alternating (P0) or serial (E13 and E15) sections were collected in 0.05 M phosphate-buffered saline (PBS), pH 7.5. Excess unreacted aldehyde was neutralized using a 30-minute incubation in 0.1 M glycine and 15 min in 0.5% sodium borohydride in PBS. After washing the tissue sections in PBS, they were incubated in a PBS blocking solution for 1 h at 4 °C containing 5% normal goat serum, 0.3% Triton X-100 (Tx), and 1% hydrogen peroxide. After the blocking step, the tissue was incubated in primary antisera containing 1% BSA and 0.3% Tx. Anti-CRH antibody was generously provided by Dr. W. Vale and used at dilutions ranging from 1:25,000 to 1:100,000. Tissue sections were incubated over 2 nights at 4 °C with primary antiserum. Sections were washed at room temperature in PBS containing 1% normal goat serum and 0.02% Tx. Sections were then incubated at room temperature in secondary antisera using a 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 further processed using a Vectastain ABC Elite kit (3 µl of reagents A and B per ml) (Vector

Laboratories, Burlingame, CA) for 1 h at room temperature. Reaction product was produced in a 5-minute incubation in Tris-buffered saline (pH 7.5) containing 0.025% diaminobenzidine (DAB) with 0.02% nickel and 0.02% H_2O_2.

To determine if changes seen in the number of immunoreactive neurons (in P0 $GABA_B$ KO vs control analysis) were due to a difference in the number of neurons expressing CRH or the amount of CRH produced in those neurons, the primary antiserum was used at two dilutions (1:50,000 and 1:100,000). The 1:50,000 dilution produced maximal signal without elevated background immunoreactivity in the PVN of newborn mice. The 1:100,000 dilution was used to maximize the dynamic range of the immunoreactive product that was generated relative to different antigen concentrations given a fixed 5 minute DAB/nickel reaction time. Tissue sections used for direct comparison to assess $GABA_B$ receptor regulation of CRH were processed in parallel in the same immunohistochemical run. Males and females were not processed together therefore comparisons were not made between the sexes. Embryonic characterization of CRH development was achieved using the CRH antiserum at a 1:25,000 dilution (McClellan et al., 2010).

Data analysis:

To assess the influence of functional $GABA_B$ receptors on immunoreactive CRH, images of the PVN were taken with a 10× objective on an Olympus BH2 microscope, an Insight QE digital camera, and Spot Advanced Software. All images used for direct comparisons were acquired in the same session with the same illumination intensity and capture time. Two independent image analysis methods were used to verify findings. In the first method we directly counted the number of CRH immunoreactive neurons after images were thresholded to 75 on a scale of 0 to 256 using MetaMorph Image analysis software (MetaMorph Offline version 7.7.1.0, Molecular Devices, Inc.). In the second method immunoreactive area was quantified in IP Lab Imaging

software (Scanalytics Inc., part of BD Biosciences, Rockville, MD) as reported previously (McClellan et al., 2010). All analysis was done with investigators blinded to genotype. Photomicrographs were normalized for optimal contrast using the “levels” tool and image quality was enhanced using the “unsharp mask” tool in Adobe Photoshop (version CS for Macintosh).

To assess the region dependent component of changes in CRH immunoreactive levels, sections containing PVN were arranged from rostral to caudal. As we sectioned at 50 µm, 6 to 7 sections per pup contained immunoreactive CRH in the PVN. Alternating sections were split between two antibody concentrations, with 3 to 4 sections per concentration containing immunoreactive CRH in the PVN. IP lab was used to quantify immunoreactivity in the most rostral section, middle section and most caudal section for each antibody concentration. In the two cases, where more than 3 sections were present, the section with the least immunoreactivity was dropped from analysis (containing no more than 3 or 4 immunoreactive cells).

To ensure that any changes in immunoreactive area were not due to alterations in cell size, CRH cell body width (perpendicular to neurite extension) was measured using MetaMorph Image Analysis Software line tool. Widths of immunoreactive CRH neurons ranged from 7.7 µm to 15.5 µm with no statistically significant differences between genotypes.

Results

Pattern of immunoreactive CRH in development:

Immunoreactive CRH peptide was first detected in the developing brain in distinct cell bodies lateral to the hypothalamus on E13 (Fig. 3.1A and B) with few cell bodies in the region of the PVN. At E15 the PVN was populated by a robust complement of CRH immunoreactive neurons (Fig. 3.1C and D) that corresponds with the developmental timing for CRH mRNA in the PVN reported previously (Keegan et al., 1994). Also at E15 (Fig. 3.1C and D) there were a number of neurons between the PVN and the region lateral to the hypothalamus where CRH neurons were visible at E13. At P0 CRH immunoreactive neurons were no longer found between the PVN and the lateral regions. Thus, while CRH immunoreactivity in the lateral regions became more diffuse and less robust, CRH immunoreactivity in the PVN increased. Weak CRH immunoreactivity was seen at the median eminence and the posterior pituitary at E15 (data not shown) that notably increased in strength at P0 (Fig. 3.2). At P0 CRH immunoreactivity clearly delineated the amygdala with a diffuse distribution of immunoreactive neurons extending from the amygdala, in apparent continuity along the stria terminalis, to the Bed Nucleus of the Stria Terminalis

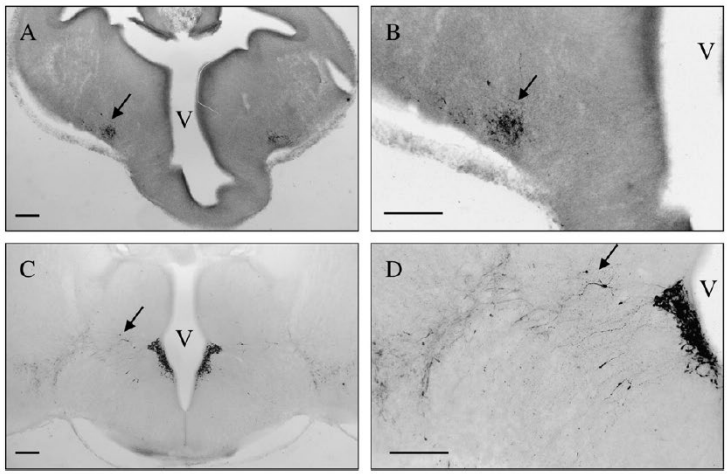

Figure 3.1. Developmental pattern of CRH IR. CRH neurons were first visible in a region lateral to the PVN (arrows) at E 13 (A and B). Later, CRH neurons populate the PVN while the lateral CRH groups becomes more difuse and CRH neurons were found between the two regions at E15 (C and D). The images in B and D are higher magnifications of the images in A and C, respectively. V, third ventricle; Scale bars = 200µm.

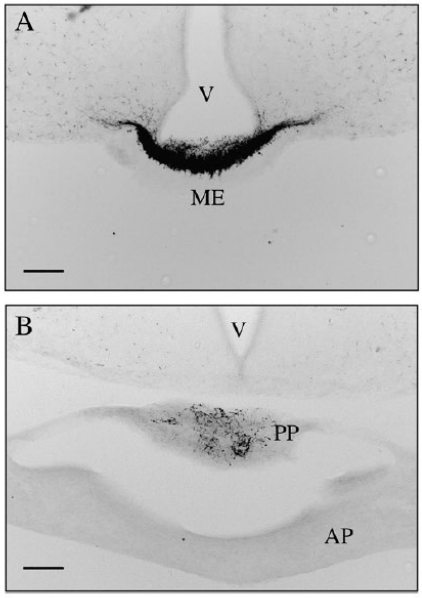

Figure 3.2. In newborn mice, CRH fibers were seen in the median eminence (A) and posterior pituitary (B). In panel B both anterior (AP) and posterior (PP) lobes of the pituitary are visible with IR limited to the posterior lobe. V, third ventricle; scale bars indicate 100μm.

(BNST). At P0, weak immunoreactive CRH neurons were also found diffusely through the preoptic area and hippocampus (data not shown).

Impact of $GABA_B$ receptors on levels of immunoreactive CRH:

On the day of birth (P0) female mice that lacked functional $GABA_B$ receptors had more immunoreactive CRH peptide than controls (Fig. 3.3). When the anti-CRH antiserum was used at a 1:100 K dilution, more than twice as many CRH immunoreactive neurons were labeled above threshold in female KO mice as compared to female control mice (thresholded cell counts: T test mean; CTRL 9.3 ± 1.3, KO 22.0 ± 3.9; N = 4, df = 6, p = 0.01 and done by immunoreactive area in pixels: T test mean; CTRL 829.8 ± 146.7, KO 1844.8 + 434.9; N = 4, df = 6, p = 0.03). There was no statistically significant difference in the number of CRH immunoreactive neurons above threshold when the anti-CRH antibody was used at a 1:50 K dilution (thresholded cell counts: T test mean; CTRL 100.3 ± 15.6, KO 121.5 ± 20.0; N = 4, df = 6, p = 0.22 and done by immunoreactive area in pixels: T test mean; CTRL 7548.5 ± 1174.8, KO 10482 ± 2637.8; N = 4, df = 6, p = 0.17). This suggests that there was no difference in the number of neurons that produce CRH peptide, but rather a change in the amount of peptide present.

Two separate experiments were done for males (N = 3 for each). There was more variability seen in male data compared to female data and neither of the individual experiments yielded statistically reliable differences in CRH immunoreactivity above threshold when quantified by

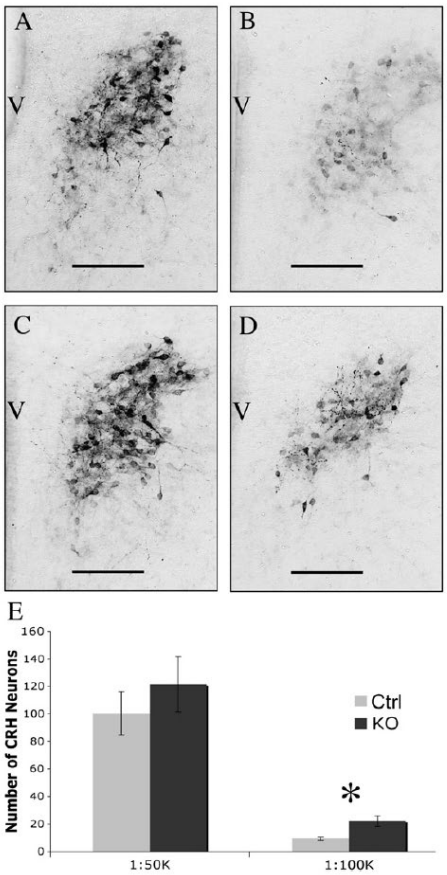

Figure 3.3. $GABA_B$ receptor regulation of newborn female CRH IR in the PVN. P0 female mice that lacked functional $GABA_B$ receptors (C and D) had more CRH peptide than control littermates (A and B). There was no statistically significant difference seen when the antiserum was used at 1:50K dilution (A and C) while knockouts (D) had more then twoce as many CRH neurons compared to control (B) when the antiserum was used at a 1:100K dilution. Data are neuron counts in images after thresholding in metaMorph and are displayed graphically in panel E. There were 4 animals in each group. Error bars represent SEM, scale bars indicate 100µm; V, third ventricle; *, $p<0.05$.

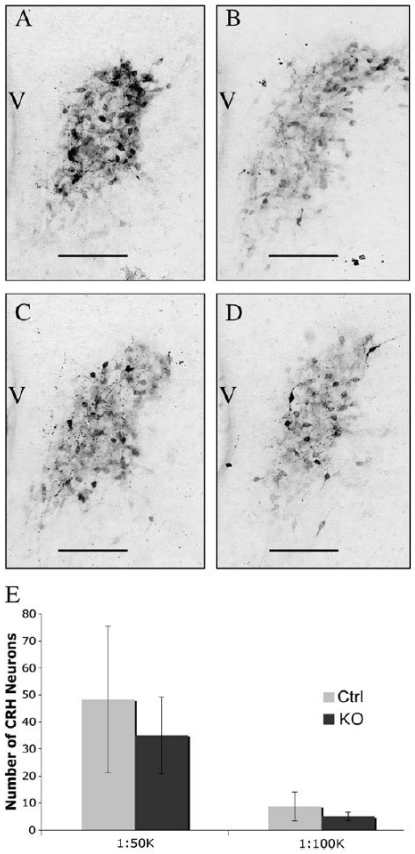

Figure 3.4. Lack of $GABA_B$ receptor regulation of newborn male CRH IR in the PVN. P0 male mice that lacked functional $GABA_B$ receptors (C and D) had the same amount of CRH peptide as wild type littermates (A and B). There was no statistically significant difference between genotypes when the antiserum was used at either the 1:50K or 1:100K dilutions. Data are from neuron counts in images after thresholding in MetaMorph and are displayed graphically in panel E. There were 3 animals in each group. Error bars represent SEM and scale bars indicate 100µm; v, third ventricle.

either method (cell number or immunoreactive area; Fig. 3.4). To ensure that statistical significance was not absent due to the small sample size (3) in the individual experiments, the two separate replicates using males were normalized to each other by creating a ratio of the means, thus increasing the total sample sizes to 6. Again there were no statistically reliable differences in CRH immunoreactivity (1:50,000 immunoreactive area in pixels: T test mean; CTRL 7528 ± 1735.6, KO 9030 ± 1180.2; N = 6, df = 10, p = 0.24 and for the more dilute 1:100,000 immunoreactive area in pixels: T test mean; CTRL 1716 ± 613.2, KO 2402 ± 884.2; N = 6, df = 10, p = 0.22).

Region specific impact of $GABA_B$ receptors on immunoreactive CRH in females:

Immunoreactive CRH neurons were not uniformly distributed throughout the PVN as fewer cells were seen in caudal sections (Fig. 3.5). At the 1:50 K concentration of antiserum, there were no genotype differences in the rostral, middle, or caudal PVN sections (genotype × location as a repeated measure, $F[2,12] = 0.656$, $p > 0.05$). When images were analyzed from adjacent sections, processed at the more dilute 1:100 K, there were significantly greater levels of immunoreactive CRH above threshold in rostral sections in the $GABA_B$ receptor KOs compared to control littermates (Fig. 3.6). Rostral sections from female KO mice had three-fold more CRH immunoreactivity above threshold than rostral sections from control mice. Repeated measures analysis revealed a significant genotype × location interaction ($F[2,16] = 3.87$, $p < 0.05$). The effect was due almost entirely to a significant increase in CRH immunoreactivity above threshold in the rostral sections of KO mice compared to control based on a posthoc analysis of the 95% confidence interval for the rostral sections. There was little difference in immunoreactivity in the middle or caudal PVN sections between genotypes at the more dilute antiserum concentration.

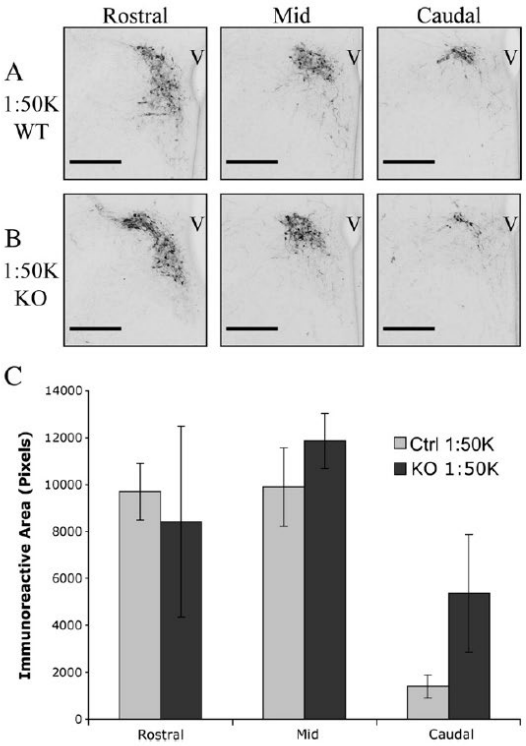

Figure 3.5. Rostral to caudal distribution of CRH in newborn female mice (1:50K antiserum dilution). There were similar levels of CRH in sections from rostral middle and caudal PVN from control (A) and knockout (B) neonates. Data is displayed graphically in panel C. There was no statistically significant interaction between genotype and locataion analyzed as a repeated measure. Error bars represent SEM and scale bars indicate 200µm; V, third ventricle.

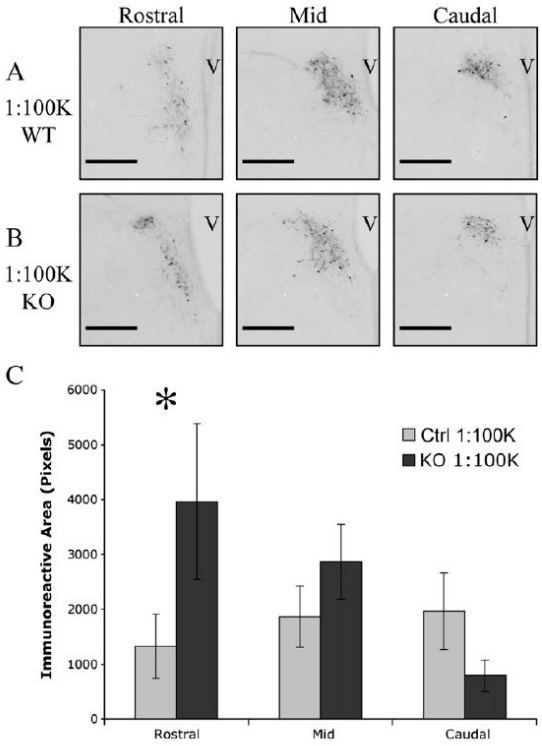

Figure 3.6. Rostral to caudal distribution of CRH in newborn female mice (1:100K antiserum dilution). Rostral sections from female KO mice (B) had three-fold more CRH than rostral sections from control mice (A). Data is displayed graphically in panel C. Repeated measures analysis revealed a significant genotype x location interaction that was due almost entirely to a significant increase in CRH in the rostral sections of KO compared to control mice ($p<0.05$). Error bars represent SEM and scale bars indicate 200µm; V, third ventricle.

Discussion

CRH is a critical peptide for the function of the HPA axis, particularly when originating from neurons in the PVN. In the current study, immunoreactive CRH was examined from the perspective of developmental time course and from the perspective of $GABA_B$ receptor regulation. Immunoreactive CRH was first visible during mouse brain development in a region lateral to the PVN, later becoming apparent in the PVN, while some CRH immunoreactive neurons were found in the region between these two locations. Whether the changing location of CRH immunoreactive cells indicates neuronal migration remains to be determined. In the absence of functional $GABA_B$ receptors, CRH immunoreactive neurons were found in the same places, but distinctive changes in CRH immunoreactivity were revealed between rostral and caudal regions of the PVN. Loss of functional $GABA_B$ receptors resulted in more CRH immunoreactivity in the rostral PVN. Interestingly, this relationship was only found in females. The results of the current study emphasize that the local relationship between a global neurotransmitter, like GABA, and a small nuclear group, like the PVN, can vary significantly as a function of sex and location.

The developmental pattern of CRH immunoreactivity in the PVN (Fig. 3.1) is suggestive of a migratory route for CRH neurons from an extra hypothalamic region into the PVN. This would be a novel model of PVN development. The changing positions of phenotypically identified cells at different ages during development are frequently associated with migration (Wray et al., 1989, Schwanzel-Fukuda and Pfaff, 1989 and Tobet et al., 1999). There is precedent for suggesting that cells migrate into the PVN from lateral regions (Urgumov, 2002; Whitnall et al., 1985). In the development of the vasopressin (AVP) system, where AVP is present in both the SON and PVN, some have hypothesized that AVP neurons migrate from the proliferative zone adjacent to the PVN to the SON, with a subpopulation reversing direction and returning to the PVN to make

up the AVP positive cells in the adult (Ugrumov, 2002). Video microscopy has revealed unexpected directions for neuron migration in different brain regions, including gonadotropin-releasing hormone containing neurons (Bless et al., 2005) and neurons that migrate ventrally in the cerebral cortex toward the ventricular zone (Nadarajaha et al., 2002). Fluorescent cells have been identified moving toward the PVN from lateral regions using video microscopy in organotypic slices dissected from E13 mice (Stratton, Searcy, and Tobet, unpublished observations).

The presence of CRH fibers in the median eminence prenatally, and in the posterior pituitary at birth (Fig. 2), indicates a potential active role for CRH regulation of early developmental events. CRH receptors, while present in large densities in the central nervous system, are also present in several peripheral organs including the spleen (Webster et al., 1990), heart (Heldwein et al., 1996), skin (Roloff et al., 1998) and adrenal gland (Muller et al., 2001). Although first reported almost 30 years ago (Bloom et al., 1982), little is known about the physiological significance of CRH immunoreactive fibers in the posterior pituitary. Immunoreactive CRH has been reported in magnocellular oxytocin neurons thought to project to the posterior pituitary (Sawchenko et al., 1984). The current data, however, provides direct evidence of immunoreactive CRH in fibers in the mouse posterior pituitary during early development.

The data in the current study indicates that cells containing immunoreactive CRH in the rostral extension of the PVN are more impacted by the loss of functional $GABA_B$ receptors than central and caudal neurons. In the rostral sections, loss of $GABA_B$ receptors resulted in significantly elevated levels of immunoreactive CRH. This suggests alternate roles for $GABA_B$ signaling on CRH expression in different regions of the PVN. Although mostly described in rat, others have shown functionally specific projections of parvocellular neurons (either to median eminence or brain stem/caudal brain structures; Geerling et al., 2010; Simmons and Swanson, 2009) and

identified protein co-expression in CRH neurons (steroid receptors; Bingham et al., 2006) based on spatial distributions within the PVN. A recent investigation of the location of neurons in the PVN that contain thyrotropin-releasing hormone in mice (Kadar et al., 2010) revealed that those TRH neurons projecting to the median eminence are differentially segregated in the PVN. Together, this indicates a high degree of compartment specific regulation of developmental processes within the PVN. Due to the fact that rostral PVN was more impacted by the loss of functional $GABA_B$ receptors, a closer examination of $GABA_B$ R1 immunoreactivity was undertaken. The $GABA_B$ receptor is present during embryonic development and enriched in the PVN, but there was no obvious rostral bias to $GABA_B$ R1 immunoreactivity in the PVN noted (data not shown).

The current data suggest a $GABA_B$ receptor dependent regulation of CRH peptide that may be sex specific. The observation that CRH immunoreactivity was altered by the lack of functional $GABA_B$ receptors only in female mice is an example of a sex-specific mechanism of protein regulation. While there are multiple examples of sex differences in protein expression in particular brain locations, there are fewer examples of sex-specific mechanisms of regulation. Interestingly, there are changes in the locations of estrogen receptor α cells in the PVN of the same line of $GABA_B$ receptor KO mice, also in the same sex-dependent manner (females only; McClellan et al., 2010). This could indicate that female mice are more dependent on $GABA_B$ receptor signaling for the development of the PVN compared to males. By contrast, there was no indication of sex dependent changes in estrogen receptor α containing cells in $GABA_B$ R1 subunit KO mice in the region of the ventromedial nucleus of the hypothalamus (McClellan et al., 2008).

It is unclear if the reported increase in CRH immunoreactivity is a primary effect of loss of functional $GABA_B$ receptors or if it is due to some other mechanism that is perturbed in the

$GABA_B$ R1 knockout. Previously reported phenotypes of the PVN in these mice include altered distribution of ERα and nNOS along with decreased BDNF immunoreactivity. Both nitric oxide (Gadek-Michalska and Bugajski, 2008; Hsieh et al., 2010) and BDNF (Givalois et al., 2004; Naert et al., 2006) have been shown to influence HPA axis activity and CRH secretion. The mislocalization of ERα in the $GABA_B$ R1 knockout might be of particular importance as estrogens act through ERα to create more robust HPA axis activity in response to stressors (reviewed in Handa et al., 2009). There is also evidence for direct estradiol regulation of CRH expression (Lalmansingh and Uht, 2008) and changes in stress response throughout the female reproductive cycle (Altemus et al., 2001).

Several other factors have also been shown to influence CRH expression at either mRNA or peptide levels. The HPA axis and CRH neurons in the PVN are often under glucocorticoid mediated negative feedback (reviewed in Watts, 2005). As noted above, sex steroid hormones have also been shown to regulate CRH expression. For the current study, however, females were taken at P0, when the ovary is thought to be relatively quiescent. Interestingly, at this point in development there is likely variability in testosterone levels in males, as testes in different males may produce different amounts of hormone, possibly explaining the increased variability seen in the current male CRH data. In addition to circulating factors, locally secreted factors (or neurotransmitters) like GABA (Cullinan et al., 2008) can influence HPA axis activity and CRH expression. GABAergic neurons innervate CRH neurons in the PVN (Herman et al., 2005; Herman et al., 2002; Miklos and Kovacs, 2002). When GABA degradation was blocked with γ-vinyl-GABA, CRH mRNA and peptide were increased in the PVN/anterior hypothalamus, but not in other brain regions (Tran et al., 1999). GABA acting through $GABA_B$ receptors in the PVN dampens HPA output in response to stress. When phaclofen was used to antagonize $GABA_B$ receptors in the PVN, stress-induced corticosterone secretion was increased (Marques de Souza and Franci, 2008). There is, however, no previous evidence to suggest that either of

these factors impact CRH expression in neonatal rodents as was seen in the current study using mice. Unfortunately, on the C57BL/6 background, $GABA_B$ R1 deficient mice die from seizures on or around P21 (Prosser et al., 2001), which makes adult HPA axis and behavior testing impossible. However, on the BALB/c background these mice survive into adulthood and show increased anxiety-like behaviors (reviewed in Cryan and Kaupmann, 2005).

There are multiple levels of regulation that might affect the amount of CRH peptide present in cells of the PVN. Protein levels can be regulated by transcriptional activity, transcript stability, translational activity, protein stability and peptide secretion. In preliminary experiments to examine CRH from an mRNA perspective, we used quantitative real-time PCR to examine CRH mRNA in $GABA_B$ R1 knockouts at P0. The preliminary data indicated a potential 50% decrease in the copy number of CRH mRNA in female knockouts compared to wild type littermates (T-test, n = 3(control), 4 (KO); p = 0.03). Thus, the increase in apparent immunoreactive content might be independent of gene transcription regulation. Given the complexities of peptide regulation, it is difficult to determine the precise physiological consequences of these differences. Nonetheless, the preliminary mRNA data and the immunohistochemical results suggest that the loss of functional $GABA_B$ receptor signaling impacts the physiological handling of CRH.

Increased amounts of CRH mRNA, CRH peptide and CRH containing cells have been associated with human depression (Raadsheer et al., 1994; 1995). When looking at the downstream effects of long term HPA axis activity it is interesting to note that disorders resulting from dysregulation of the HPA axis like major depressive disorder and anxiety related disorders, are more prevalent in females than males. Our data that females but not males lacking functional $GABA_B$ receptors have more CRH peptide in the PVN might help to explain some of the predisposition of disorders resulting from HPA axis dysregulation in females. That is to say,

if a mutation or insult affecting the $GABA_B$ signaling system resulted in altered HPA axis activity and behavior it might preferentially impact females.

CHAPTER 4. EMBRYONIC $GABA_B$ RECEPTOR BLOCKADE ALTERS CELL MIGRATION, ADULT HYPOTHALAMIC STRUCTURE, AND ANXIETY- AND DEPRESSION-LIKE BEHAVIORS SEX SPECIFICALLY IN MICE

Summary

Neurons of the paraventricular nucleus of the hypothalamus (PVN) regulate the autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. Previous experiments have shown that mice lacking functional $GABA_B$ receptors have sex specific alterations in PVN cell placement and protein expression and altered anxiety-like and depression-like behaviors. The current work tested whether alterations in $GABA_B$ receptor signaling that influence cell movements in the developing PVN also might cause lifelong changes in PVN cytoarchitecture and physiology and behaviors related to HPA axis function. Fluorescence video microscopy of fetal mouse organotypic brain slices revealed a role for $GABA_B$ receptor signaling in regulating neuron movements selectively in slices from females. Movement speeds of cells in the region of the developing PVN in slices from females, but not males, increased significantly 52% over baseline following $GABA_B$ receptor antagonism. Maternal treatment with a $GABA_B$ receptor antagonist CGP 55845 resulted in offspring with female-selective alterations in the placement of cells containing estrogen receptor α in the region of the PVN and anxiety-like behavior (elevate plus maze) in adulthood. Males and females displayed decreased depression-like behaviors (tail suspension and sucrose preference) and decreased restraint stress induced HPA axis activation (immunoreactive FOS in PVN and plasma corticosterone). This work highlights the importance of $GABA_B$ signaling for PVN development and the expression of multiple complex behaviors in adulthood. The replication of cytoarchitectural and behavioral phenotypes of $GABA_B$ receptor knockout mice by antagonist treatment during a critical period of development emphasizes the role of GABA in a key fetal antecedent mechanism.

Introduction

The prevalence and impact of many disorders often varies as a function of sex (Viveros et al., 2012). Agoraphobia and General Anxiety Disorder impact 4 and 3 times more females than males, respectively (Bekker and van Mens-Verhulst, 2007), while all forms of Attention Deficit Hyperactivity Disorder (ADHD) are more prevalent in males than in females (Biederman et al., 2002). Major Depressive Disorder (MDD) also preferentially impacts females is with almost 2 times more females affected than males (Kessler et al., 1993).

Dysregulation or hyperactivity of the hypothalamic pituitary adrenal (HPA) axis has been implicated in both of the female predominant disorder types listed above, anxiety and depression (Mello et al., 2003). The final common pathway for all brain circuits influencing the HPA axis is the paraventricular nucleus of the hypothalamus (PVN). The PVN has been extensively studied and a number of reviews of its structure, function and regulation are available (Swanson and Sawchenko, 1983; Tasker and Herman 2011; Pyner, 2009).

We previously discovered female specific alterations in cell placement and peptide content in and around the PVN of newborn mice that lack functional $GABA_B$ receptors (McClellan et al., 2010; Stratton et al., 2011). There are unique patterns of immunoreactive GABA, GAD 65/67 and $GABA_B$ R1 subunits in the developing mouse hypothalamus. GABA and GAD surround the developing PVN with little or no immunoreactivity in the PVN (McClellan et al., 2010). At the same time, GABA receptors including $GABA_B$ are enriched within cells of the developing nucleus (McClellan et al., 2010).

The evidence for altered GABA signaling contributing to depression and anxiety disorders is growing (reviewed; Kalueff and Nutt, 2007; Luscher et al., 2011; Ghose et al., 2011). There are

decreased GABA concentrations in the plasma (Prosser et al., 1997), CSF and brain (Sundman-Eriksson and Alard, 2002; Kugaya et al., 2003; Epperson et al., 2006) and fewer GABA neurons in the orbitofrontal cortex of individuals with various forms of depression. Also, there is a link in polymorphisms of the GAD 65 gene with anxiety behaviors in children (Smoller et al., 2001). In animal models, disrupting the GAD genes (Kash et al., 1999; Stork et al., 2000), $GABA_A$ (Crestani et al., 1999), or $GABA_B$ (Mombereau et al., 2004; Gassmann et al., 2004) receptors all cause anxiety-like phenotypes and some have been reported to cause changes in depression-like behavior. In these cases, even though the given mutation is present throughout development and the chemical imbalances are likely present throughout development, results have been interpreted from the standpoint of GABA acting in adults to regulate neuronal excitability without investigation of developmental processes.

Earlier studies on the role of GABA during hypothalamic development utilized genetically disrupted mice (Dellovade et al., 2001; McClellan et al., 2008; 2010) for which the disruptions were permanent. To determine fetal antecedents of adult disorder, it is necessary to show that manipulations that are restricted to fetal development have long-term consequences. The current studies were conducted to determine influences of disrupting $GABA_B$ receptor signaling during a critical window of embryonic development.

Materials and Methods

Animals:

Founder mice from the *B6.Cg-Tg(Thy1-YFP)16Jrs/J* transgenic mouse line (Feng et al., 2000) were obtained from The Jackson Laboratory and were used for fluorescence video microscopy as described previously (Tobet et al., 2003). The Thy-1 promoter was used to drive YFP neuronal expression in the brain, including the developing PVN. The pattern of YFP expression

allowed for tracking of individual immature neurons in the developing hypothalamus. Females were checked for vaginal plugs the following morning. The day of plug was designated as E0 and mice were taken at embryonic day 13 (E13).

To generate animals with decreased GABAB receptor activation during a specific fetal period of PVN development, time mated pregnant dams (C57BL/6J) were injected (s.c.) with 0.1mg/kg CGP 55845 (LaSarge et al., 2010) (Tocris Biosciences) or vehicle (1% DMSO, 0.25% EtOH, PBS) daily from E11 thru E17. These animals were weaned at postnatal day 19, assigned random numbers and marked by ear punch. Animals were housed in same sex cages with two to five animals per cage. Mice were maintained in plastic cages with aspen bedding (autoclaved Sani-chips, Harlan Teklad, Madison, WI, USA) in the Painter Building of Laboratory Animal Resources at Colorado State University. Food (# 8 640, Harlan Teklad, Madison, WI, USA), tap water, and environmental enrichment were provided ad libitum in a 14/10 h light/dark cycle.

After the completion of behavior testing, animals were deeply anesthetized and perfused transcardially with 10ml heparin PBS followed by 10ml neutral-buffered 4% paraformaldehyde (0.1M PB, pH 7.4). Brains were postfixed overnight in 4% paraformaldehyde and changed into 0.1M phosphate buffer (PB) the following day. Brains were stored in 0.1M PB at 4°C until used for immunocytochemistry. 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.

Fluorescence Video Microscopy:

Embryos were harvested at E13, a developmental age when the majority of cells destined to become part of the PVN have past their last mitotic event but have not yet completed their migration. Pregnant dams were deeply 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 and tissue was taken for genotyping by PCR. Sex determination was made through PCR analysis for the Y-chromosome *sry* gene (McClellan et al., 2008). Brains were dissected free 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. Live slice preparations followed previously published methods (Tobet et al., 2003; McClellan et al., 2008). Briefly, dissections were limited to a maximum of two hours to minimize cellular damage. Following each dissection, brains were embedded in 8% low gelling temperature agarose (Sigma-Aldrich) 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 PVN were chosen for video microscopy and were transferred to media (Hybernate-E; GIBCO-Invitrogen Corporation, Carlsbad, CA), with 2% pen-strep, and incubated at 36°C with 5% CO_2 for 30 minutes. Following the 30-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 Purecol (Advanced Biomatrix, San Diego, CA) and water. The slices were put back into the 36°C incubator for up to 30 minutes to promote adherence. To prevent slice movement during video observation, 40µL of a Purecol solution (1mL Purecol, 125µL 10XMEM, 23µL penicillin/streptomycin, and 33µL 1M sodium bicarbonate) was placed over each slice. The slices were then placed in the 36°C incubator for up to 1 hour to allow the Purecol to polymerize. 1mL of Neurobasal media (10% L-glutamine, 2% B-27, 1.1% glucose, 2% pen-strep, 2% glutamine) 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 2 days post plating.

In preparation for video microscopy, slices were washed with warm Neurobasal media (GIBCO-Invitrogen), and placed on a heated stage maintained at 37°C with 5% CO_2 and fresh

Neurobasal medium in the dish. All data was collected on either an inverted Nikon TE2000-U (Nikon USA, Melville, NY) microscope with a 20x plan Apo phase objective or an upright Olympus BX61W (Olympus America, Center Valley, PA) microscope with an XLUM PLAN FLN-W 20x 1.0 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 10µm 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 $GABA_B$ receptor antagonists, 2-hydroxy saclofen (20µM, Sigma-Aldrich, St. Louis, MO) or CGP 55845 (1µM, Tocris Biosciences, Bristol, UK). The concentrations used were chosen based on the intent to block GABA signaling through its receptors in live brain slices (Mann et al., 2009 and Kerr et al., 1988). Once a drug was administered to the slice, an additional 1.5 hours of video was taken to compare treatments. Cells were tracked with Metamorph track point tool and speeds were quantified using xy coordinates in an excel macro (Tobet et al., 2003). For a cell to have been analyzed, it must have remained in frame and focus for the duration of the experiment and have demonstrated measurable movement (at least two cell body diameters). To ensure that changes in movement speed were not due to vehicle addition, aging of the slice, or fluorescence excitation, a subset of slices was treated with vehicle in place of antagonist. Also, immunohistochemical detection of vasopressin and corticotropin releasing hormone was conducted on imaged sections posthoc to ensure that sections chosen for fluorescence video microscopy contained PVN.

Immunohistochemistry:

Brain tissue was 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), pH 7.5. Excess unreacted aldehyde 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 for 60 minutes at 4°C in a PBS blocking solution 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 antiserum was used at a 1:10,000 dilution (rabbit anti-ERα; C1355, Upstate Biotechnology, Charlottesville, VA) or a 1:5,000 dilution (rabbit anti-cFOS; sc-52, Santa Cruz Biotechnology, Santa Cruz, CA). 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 Vectastain ABC Elite kit (3 µl/ml reagents A and B) (Vector Laboratories; Burlingame, CA) for 1 hour at room temperature. After 1h of washing with Tris-buffered saline (pH 7.5), the sections were incubated for 5 minute in a substrate solution containing 0.025% diaminobenzidine with 0.02% nickel and 0.02%H_2O_2 diluted in Tris-buffered saline to produce a dark purple/black reaction product.

Radioimmunoassay (RIA):

Plasma corticosterone concentrations were measured by RIA. Plasma samples were diluted 1:25 in 0.01M PBS, and corticosterone binding globulin was denatured by incubating the samples at 65°C for one hour. All samples and standards were then incubated overnight at 4°C in the presence of antiserum (MP Biomedicals, Solon OH) and [^{3}H] corticosterone (Perkin-Elmer, Boston, MA) in 0.1% gelatin dissolved in 0.01M PBS. Unbound corticosterone was removed by adding dextran-coated charcoal, which was then separated by centrifugation. Bound corticosterone was decanted into new vials, mixed with scintillation fluid, and counted using a Beckman Coulter LS6500 Multipurpose Scintillation Counter (Brea, CA). Plasma

corticosterone concentrations were determined by comparison to a standard curve (5-700 ng/ml). The intra-assay variance was 3.26%, and was determined by an internal control which was measured at regular intervals throughout the assay

Analysis:

Images of the PVN were taken with a 10× objective on an Olympus BH2 microscope with an Insight QE digital camera and Spot Advanced Software. All images were acquired in the same session with the same illumination intensity and capture time. Sections were carefully matched by an investigator that was blinded as to treatment condition to ensure that the same section (relative to rostral to caudal distribution of the PVN) was used in the analysis for each animal. Images of sections containing immunoreactive ERα were normalized for optimal contrast in Adobe Photoshop (version CS for Macintosh). Immunoreactive area was quantified in IP Lab Imaging software (Scanalytics Inc., part of BD Biosciences, Rockville, MD) as reported previously (McClellan et al., 2010). Briefly, eight 100µm (width) x 500µm (height) columns were placed over the images starting 100µm lateral from the edge of the ventricle (to eliminate the impact of the immunoreactive artifact edge effect) and 200µm above the top of the ventricle. Immunoreactivity from each column was calculated. Analysis was blinded through a random alpha numeric coding system. For FOS analysis, an investigator blinded to treatment group manually counted FOS immunoreactive cell nuclei in the PVN (both left and right sides) from two sections per animal in the middle of the rostral to caudal extent of the PVN that were the most intensely immunoreactive sections.

For analysis of ERα immunoreactive distribution, significance was determined by three way ANOVA analyzing Sex x Treatment x Location (columns as a repeated measure) with SPSS software (SPSS Inc., Chicago, IL). Statistical significance for all other experimental endpoints

was determined by two way ANOVA analyzing Sex x Treatment, or if no sex dependence was present, with a one-way ANOVA for Treatment, also with SPSS. Once an overall significance was established in the ANOVA (e.g. significant Sex x Treatment interaction), individual relationship significance was established posthoc by non-overlapping 95% confidence intervals as appropriate. All data are expressed as mean +/- SEM.

Behavior Testing:

Behavior testing was started when mice were approximately 12 weeks of age. Females were tested while in estrus as determined by primarily cornified epithelial cells in the vaginal cytology. Animals were handled for two weeks prior to the start of testing and for at least two days before each individual test. The testing order was Elevated Plus Maze, Open Field, Tail Suspension, Forced Swim, and Sucrose Preference for all animals. Tests were accomplished during the same 4 hour window each day (11:00 am – 3:00 pm) in the light phase except for sucrose preference, which was conducted over 8 days. Tests were spaced at least 4 days apart for each animal. During each testing day the order of animal testing i.e. sex and treatment was randomized. All testing apparatuses were washed once with 10% ethanol solution and once with water then dried between animals. During all testing sessions the investigator remained in an outer anteroom and the animal was left alone in the actual testing room which was approximately thirty feet from the housing room on the same hallway in the CSU animal facility. The same investigator conducted all tests and was careful to be free of any fragrances. All tests were digitally video recorded using a Sony HD Handicam and analyzed on video playback with the assistance of the Stopwatch+ program (Center for Behavioral Neuroscience; Atlanta, GA). Analysis was blinded through a random animal numbering system. Thirty-six animals were behavior tested and each group consisted of offspring from at least three different nulliparous dams.

Elevated Plus Maze:

To determine if animals treated embryonically with CGP 55845 displayed altered anxiety-like behavior, the elevated plus maze test was used as previously described (Weiser et al., 2009A; Teegarden and Bale 2007). The dimensions of the apparatus were; lane width 5 cm, arm length 35 cm, wall height 15 cm, leg height 40 cm (Stoelting, Wood Dale, Illinois). Animals were placed at the center of the EPM facing the open arm. The testing period was 5 minutes. Arm entries and durations were quantified.

Open Field:

The open field test has been previously used as a measure of anxiety-like behavior and general activity (Oyola et al., 2012). The open field test in this experiment was conducted in a clear plastic storage bin with base dimensions of 30 cm x 37.5 cm. This was somewhat smaller than the typical dimensions of an open field test box and therefore less appropriate for analyzing anxiety-like behavior by time spent on the perimeter of the box vs time spent in the center region. Endpoints, however, were quantified indicating general activity. The testing period was 10 minutes. For analysis of the open field test the Anymaze (Stoelting, Wood Dale, Illinois) software program was used to measure mobility, immobility, and latency to immobility.

Tail Suspension:

The tail suspension test was used to measure despair-like behavior and was conducted as described previously (Goel and Bale, 2008). Briefly, mice were suspended by their tails, which were attached with adhesive tape (~ 1 cm from tip of the tail) to a horizontal rod. The testing period was 5 minutes. Times spent struggling or immobile were quantified. In several instances mice were able to climb their tails and were not counted in this analysis.

Forced swim:

As another measure of despair-like behavior, mice were tested in a version of the forced swim test modified for testing mice (Bale and Vale, 2003). Animals were placed in the cylinder for a pretest exposure for 6 min the day prior to testing and then monitored during another 6 min test on the actual testing day. The time spent swimming and immobile were quantified using Anymaze (Stoelting, Wood Dale, Illinois) analysis of video recordings by an investigator blinded to sex and treatment. Females were subjected to the pretest exposure during proestrus and tested the following day while in estrus as confirmed by vaginal cytology.

Sucrose Preference:

To assess the impact of embryonic CGP 55845 treatment on anhedonic-like behavior, the sucrose preference test was conducted as previously described (Mueller and Bale, 2008, Poulter et al., 2010) with minor modifications. Briefly, mice were moved to individual static caging to allow for the presence of two drinking bottles. Animals were allowed to acclimate to drinking from two bottles for two days (both bottles water). Then animals were acclimated to drinking 1% sucrose (w/v) for two days (both bottles 1% sucrose). On the 5^{th} day animals were given a choice of water or 1% sucrose. Bottles were changed each day at the same time of day and weighed before being put in the cage and after removal (measured 24 hours of consumption). This was repeated three times to acquire four days of consumption preference data (one bottle water one bottle 1% Sucrose). The side that contained sucrose was randomized on the first day and alternated each subsequent day.

HPA axis activation:

To determine if fetal CGP 55845 treated animals showed altered HPA axis response to stress in adulthood, corticosterone levels were assayed from serum samples before and after restraint stress. Again, females were tested in estrus as verified by vaginal cytology. Blood was

collected in heparinized tubes from the tail at the initiation restraint stress (time 0 – completed within 3 minutes of initial handling) after 30 minutes of restraint (time 30) and trunk blood was collected 60 minutes after release from restraint (time 90) just prior to perfusion fixation. Animals were anesthetized using ketamine (80mg/Kg) and xylazine (8mg/Kg) 5 minutes prior to (time 85) the final blood collection and perfusion. Samples were centrifuged at 1300 RCF for 10 minutes and serum was transferred to a new tube and stored at -80C until use. The presence of immunoreactive FOS was also used as an indicator of neuron activation in the PVN following restraint stress (see Analysis and Immunohistochemistry sections above).

RESULTS

$GABA_B$ receptor blockade and migration:

To test the hypothesis that GABA acts on immature neurons in the developing PVN through $GABA_B$ receptors to restrict movement and keep them within the PVN, two experimental designs were used. First, the acute effect of $GABA_B$ receptor antagonists on cell movements were assayed with fluorescence video microscopy in fetal organotypic brain slices. Then the positions of neurons containing ERα were determined in neonatal and adult mice that received either vehicle or $GABA_B$ receptor antagonist treatment (CGP 55845, 0.1mg/kg) during a fetal period when neurons in the developing PVN are finding their way from the proliferative zone along the ventricle to their permanent location. Previous work demonstrated that mice with disrupted $GABA_B$ receptor subunit R1 have altered cell placements in and around the PVN when examined at birth (McClellan et al., 2010).

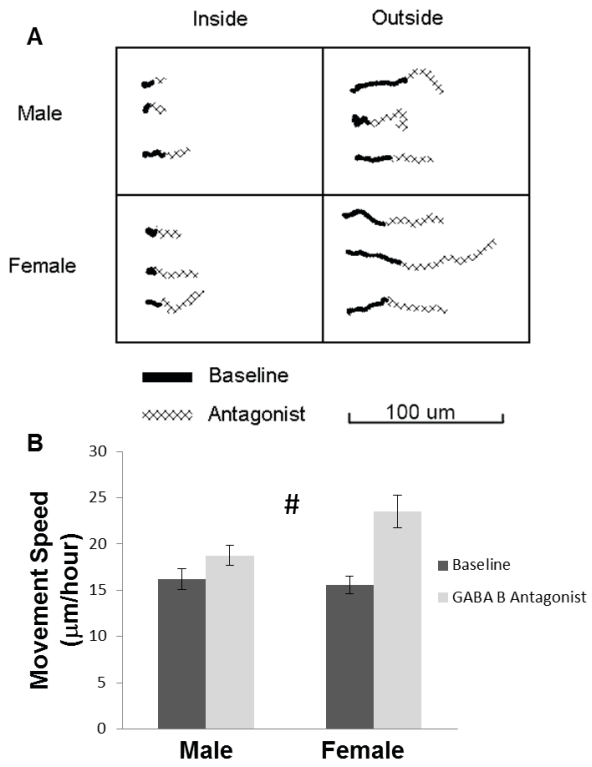

Figure 4.1. Effects of $GABA_B$ receptor antagonists 2 Hydroxy Saclofen and CGP 55845 on cell movements in ex vivo brain slice cultures. Panel A depicts representative traces of analyzed cell movements in and around the developing PVN. Solid lines represent movement over 90min of baseline and hatched lines represent movement over 90min of exposure to antagonist for the same cell in each case. Generally, cells found to be just outside of the prospective boundary of the developing PVN moved faster than cells inside of the prospective PVN (inside 13.9 +/- 0.8 μm/h, outside 18.9 +/- 1.4 μm/h at baseline, $p<0.05$). $GABA_B$ receptor antagonists caused cells in slices from female embryos, but not male embryos, to increase their rates of movement. The data is summarized by sex and treatment in panel B, illustrating a significant effect of antagonists on rate of motion only for cells from slices taken from female embryos (# indicates sex x treatment interaction, $p < 0.05$).

Ex Vivo Cell Movements:

In organotypic slices from female embryos, neurons expressing YFP under control of the Thy-1 promoter increased their cell movement speeds after treatment with either 2 hydroxy saclofen or CGP 55845. The speed of cell movement increased significantly 52% over baseline (Sex x Treatment ANOVA $F(1,82)=5.56$, $p<0.05$, n = 50 cells from 14 slices, Figure 4.1). However, cells in slices from the PVN region of male brains showed no significant changes in movement speed after antagonist treatment (male baseline = 16.21 μm/hr +/- 1.16 male antagonist = 18.7 μm/hr +/- 1.08, n = 34 cells from 16 slices). Cell movement speeds were affected similarly regardless of which $GABA_B$ receptor antagonist was used (1μM CGP 55845 or 20μM 2-hydroxy saclofen; Speed x Drug ANOVA $F(1, 80)=1.27$, $p=0.26$). Vehicle treatment did not alter cell movement speeds. A representative assembly of movement tracks can be found in Figure 4.1A.

In Vivo Cell Placement: Cells containing immunoreactive ERα were present in the PVN and in the perifornical area recently considered as the juxtaparaventricular region (Hahn and Swanson, 2010). Previously, the placement of these cells was sensitive to genetic ablation of the $GABA_B$ receptor R1 subunit as cells were shifted from the PVN to the perifornical area in newborn females (McClellan et al., 2010). Here, $GABA_B$ receptors were antagonized embryonically with CGP 55845 during a period when the PVN is developing (treatment E11-E17). A pilot experiment examined the placement of ERα containing cells at birth following embryonic treatment with CGP 55845. The results provided a positive basis (i.e., altered cell positions) to continue raising the cohort of mice that were tested for behavior and ultimately analyzed for immunoreactive ERα as presented below. When examined in adulthood, fetal treatment with the $GABA_B$ receptor antagonist CGP 55845 reproduced the phenotype seen previously in the $GABA_B$ R1 subunit knockout mouse (McClellan et al., 2010). Females showed a shift in the distribution of ERα immunoreactive cells from the PVN to the perifornical area. In the most medial column quantified, females treated with CGP 55845 had 71% less immunoreactivity than

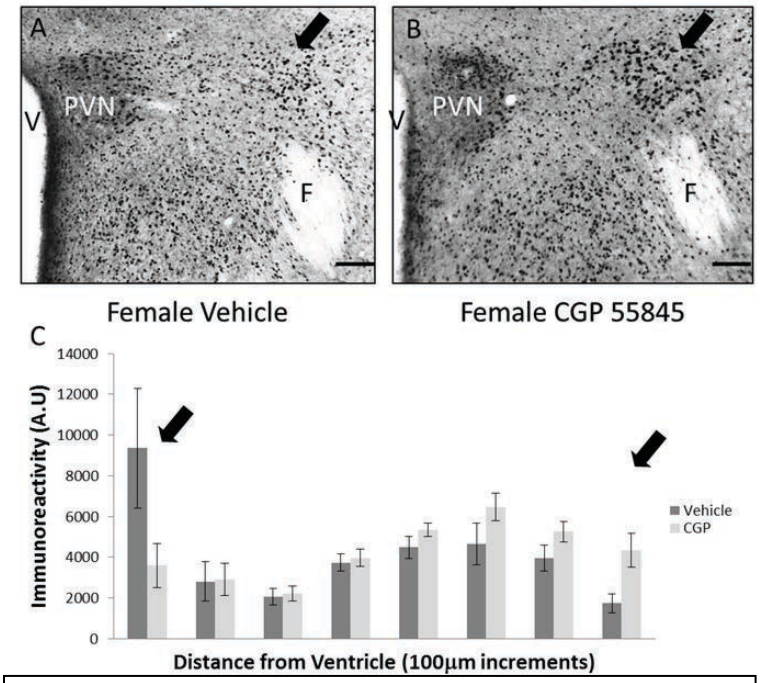

Figure 4.2. Distribution of cells containing immunoreactive ERα in female mice. In female offspring of mothers treated with vehicle (Panel A, C) a large number of immunoreactive cells were concentrated close to the third ventricle (V). By contrast, when mothers were treated with CGP 55845 female offspring (Panel B, C) had fewer cells noted in medal positions and more cells were located in positions lateral to the paraventricular nucleus (PVN) and closer to the perifornical area (black arrows). The distribution of immunoreactive cells relative to distance from the third ventricle (V) is shown quantitatively in panel C. Post hoc analysis revealed that CGP 55845 selectively shifted this immunoreactivity (medial to lateral indicated by black arrows panel C) in females ($p<0.05$). F = fornix, scale bars

vehicle treated animals while in the two most lateral columns quantified, females treated with CGP 55845 had 60% more immunoreactive area than vehicle treated animals (Sex X Treatment X Location (as a repeated measure) $F(7,154)=3.20$, $p<0.05$; n= 6 veh male, 7 CGP male, 6 veh female, 7 CGP female) (Figure 4.2). This shifted pattern was not found in males exposed embryonically to CGP 55845 (Figure 4.3). To determine to what extent the significant 3-way interaction was due selectively to females versus males, orthogonal contrasts were run for each sex individually in a Treatment X Location (again as a repeated measure) analysis. In this post hoc analysis there was statistical significance in females ($F(7,154)=5.28$, $p<0.01$), but not males ($F(7,154)=0.46$, $p=0.86$).

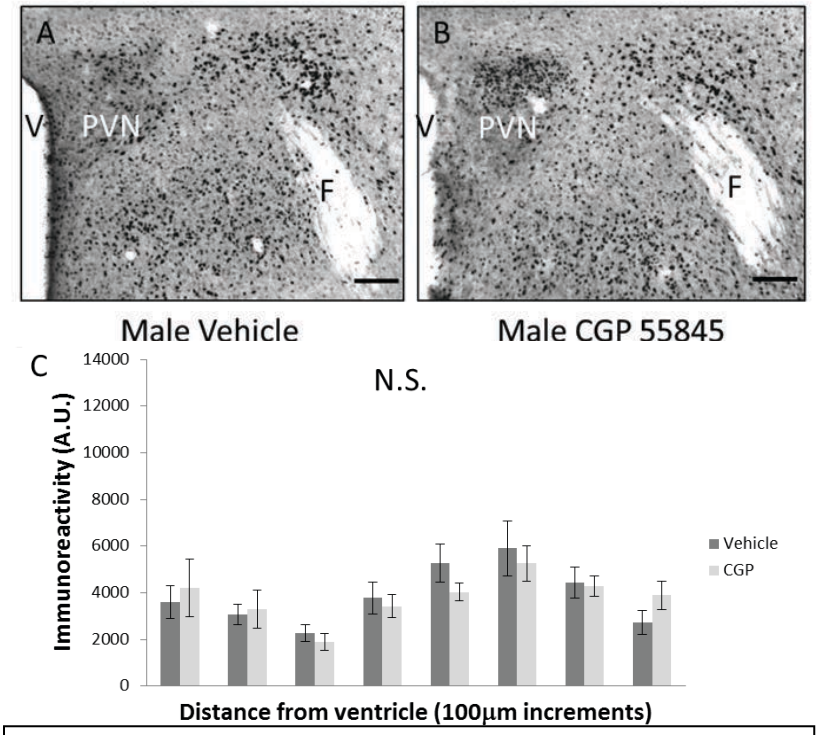

Figure 4.3. Distribution of cells containing immunoreactive ERα in male mice. In male offspring of mothers treated with vehicle (Panel A, C) cells containing immunoreactive ERα were spread relatively uniformly from the third ventricle (V) to the fornix (F). When mothers were treated with CGP 55845 male offspring (Panel B, C) had no difference in the position of immunoreactive ERα containing cells (B) relative to vehicle treated controls (A) The distribution of immunoreactive cells relative to distance from the third ventricle is shown quantitatively in panel C. Scale bars represent 200µm

Behavioral changes following embryonic $GABA_B$ receptor blockade:

To determine possible effects of embryonic $GABA_B$ receptor blockade on anxiety-like behaviors, animals were tested in the elevated plus maze. Female offspring showed significantly increased anxiety-like behavior after fetal CGP 55845 treatment as indicated by decreased time in the open arms, with the ratio of time spent in open arm divided by

time spent in any arm being half that of vehicle treated animals (Figure 4.4). Male offspring of fetal CGP 55845 treated mothers showed decreased anxiety-like behavior as indicated by increased time in the open arms (Veh = 0.11 +/- 0.02, CGP = 0.23 +/- 0.04; Figure 4.4). The sex difference in the effect of fetal CGP exposure was established by a two way Sex x Treatment ANOVA ($F(1,31)=12.50$, $p<0.05$, n = 9 veh male, 7 CGP male, 11 veh female, 9 CGP female).

Interestingly in males, the increased time in the open arms with fetal CGP treatment was accompanied by a trend toward an increased total number of arm entries with fetal CGP treatment (entries Veh = 12.4 +/- 0.86, CGP = 15.6 +/- 1.2). Overall, females were more active than males in the EPM as indicated by more arm entries (female vehicle = 16.0 +/- 1.2, male vehicle = 12.4 +/- 0.86 ($F(1,31)=4.4$, $p<0.05$)).

The apparent increase in male hyperactivity with embryonic $GABA_B$ receptor blockade was confirmed by testing in an open field. Total time spent mobile, latency to immobility and number of immobile episodes were quantified in Anymaze. Males treated embryonically with CGP 55845 showed significantly increased latency to immobility and significantly decreased immobile episodes (Figure 5; latency in seconds: male veh = 45.1 +/- 12.8, male CGP =

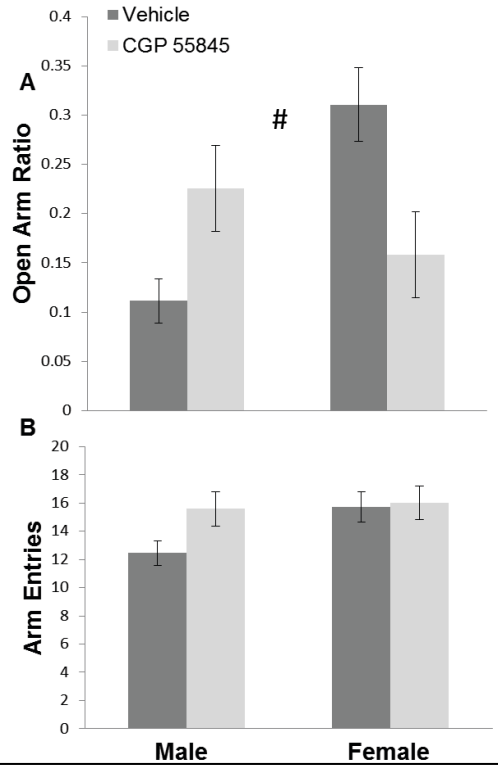

Figure 4.4. Elevated plus maze test for anxiety-like behavior: Panel A depicts the open arm ratio (time in open arms divided by time spent in any of the four arms, while panel B depicts the total number of arm entries. Female offspring of mothers treated with CGP 55845 spent less time in the open arm compared to vehicle controls. Male offspring of mothers treated with CGP 55845 showed increased open arm ratio and an increased trend in number of arm entries. Data are Mean +/- SEM. (# indicates sex x treatment interaction, $p < 0.05$)

125.9 +/ - 29.2 Sex X Treatment two way ANOVA $F(1,32)=7.57$, $p<0.05$, n = 9 veh male, 7 CGP male, 11 veh female, 9 CGP female) (episodes: male veh = 23.7 +/- 2.5, male CGP = 11.7 +/- 2.8, Sex X Treatment two way ANOVA $F(1,32)=12.4$, $p<0.05$). There was a 45% decrease in time spent immobile in males treated with CGP 55845 relative to controls that did not reach statistical significance due to the variability (immobile time male veh = 98.9 +/- 10.7, male CGP = 53.9 +/- 16.6). There was no indication of an effect of fetal CGP treatment on any female activity in the open field test.

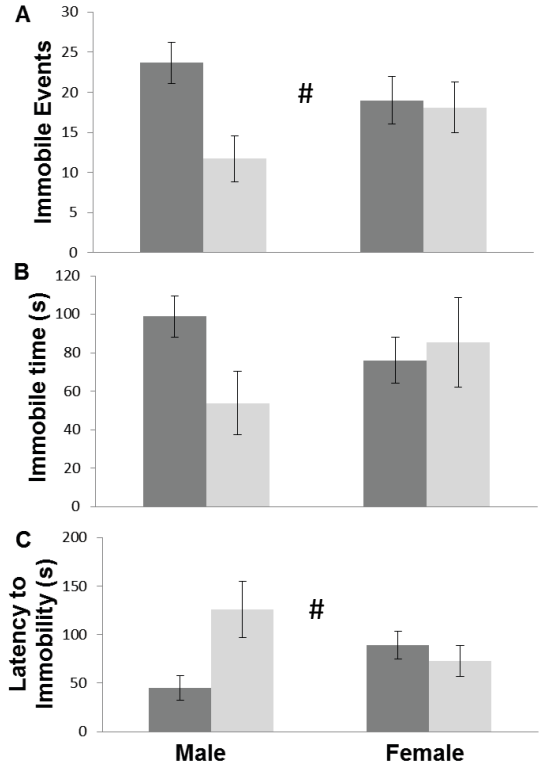

Figure 4.5. Graphs depict measurements of activity in an 'open field'. Number of immobile events (A), time spent immobile (B) and latency to immobility (C) were quantified. Male offspring from mothers treated with CGP 55845 during pregnancy displayed a hyperactivity-like phenotype that was most obvious for the number of immobile events (A) and the latency to immobility (C). Males also showed decreased immobility with embryonic exposure to the $GABA_B$ receptor antagonist although this did not reach statistical significance (B). There were no obvious treatment effects on the activity of females. # indicates $p < 0.05$ for the interaction between sex and treatment. Data are Mean +/-SEM.

To determine possible effects of embryonic $GABA_B$ receptor antagonism on depressive-like behaviors the tail suspension, forced swim and sucrose preference tests were used (Figure 4.6). In the tail suspension test, fetal CGP 55845 treatment resulted in decreased depressive-like behavior in males and females as indicated by decreased immobile time and increased struggling in adulthood. In females, time spent immobile was 66% less (fem veh = 123.0 +/- 9.7, fem CGP = 73.9 +/- 10.9 with fetal CGP treatment while in males immobile time was 14% less (male veh = 147.7 +/- 11.2, male CGP = 129.2 +/- 13.4). There was a significant effect of fetal treatment based on the main effect ($F(1, 27)=8.67$, $p<0.05$). As the Sex X Treatment interaction was not statistically

significant (F(1, 27)=1.79, p=0.19 n = 6 veh male, 7 CGP male, 9 veh female, 9 CGP female), this measure suggests that both male and female offspring were similarly effected by fetal treatment with CGP 55845. Three males and two females climbed their tails and were excluded from this analysis on that basis. Interestingly, tail climbing was found only in the vehicle treated group. There was no effect of embryonic exposure to the $GABA_B$ receptor antagonist on immobility in the forced swim test. It has been previously shown that in some instances the TST and FST have different sensitivities to altered behavior in mice, with some compounds showing activity in one test but not the other (Cryan et al., 2005).

In the sucrose preference test, males and females displayed decreased anhedonia-like behavior as indicated by increased preference for 1% sucrose solution over water. The ratio of sucrose consumption to water consumption increased 70% from 1.84 for vehicle treated animals to 3.18 for CGP treated animals. Statistical analysis showed a significant main effect of treatment F(1,31)=6.9, p<0.05, without a significant Sex X Treatment interaction F(1,31)=0.81, p=0.375, n = 9 veh male, 7 CGP male, 11 veh female, 9 CGP female).

Adult animal weights were also analyzed.

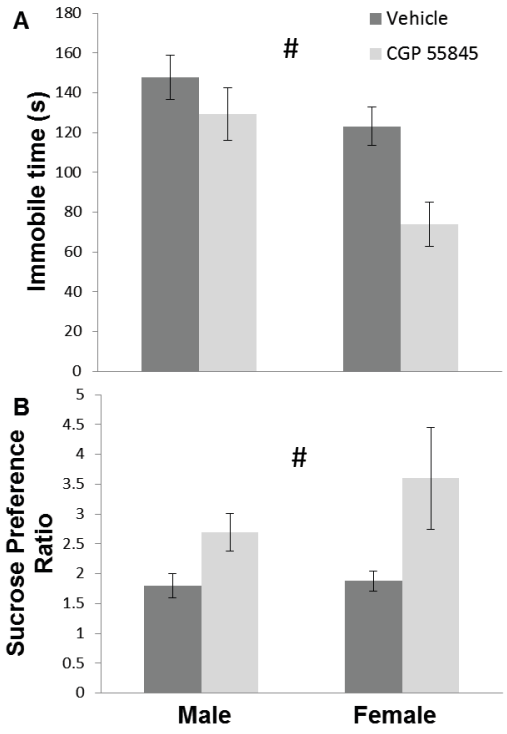

Figure 4.6. Tests for depression-like behaviors: Panel A depicts the results of tail suspension tests, while Panel B depicts the results of sucrose preference testing. For the tail suspension test (A) both male and female offspring of mothers treated with CGP 55845 during pregnancy showed decreased immobility. For the sucrose preference test (B) both male and female offspring exposed to fetal CGP 55845 showed increased sucrose preference. The main effect of treatment was statistically significant as indicated by the symbol # in both panels ($p < 0.01$), while the sex x treatment interaction was not ($p > 0.10$). Data are Mean +/- SEM.

Although there was a trend for female offspring of mothers injected with CGP 55845 to be heavier (female vehicle = 19.4 +/- 0.4g, female CGP 55845 = 20.5 +/- 0.4g), sex by treatment ANOVA did not yield a significant interaction. Males did weigh more than females regardless of treatment (male = 25.6 +/- 0.3g, female = 19.8 +/- 0.3g, $F(1,32)=233.8$, $p<0.01$).

HPA axis activation:

One hour after release from restraint stress, neuronal activation in the PVN as indicated by immunoreactive FOS was markedly reduced 31% in animals that experienced fetal $GABA_B$ receptor pharmacological blockade (Figure 4.8). This occurred similarly in males and females (main effect of treatment $F(1,12)=8.5$, $p<0.05$; Treatment x Sex interaction $F(1,12)=0.691$, $p=0.422$) n=4 veh male, 4 CGP male, 4 veh female, 4 CGP female).

At the start of restraint stress (within 3 minutes of initial handling/time 0), female corticosterone values were significantly higher than in males (male = 10.3 +/- 3.5, female = 24.6 +/- 3.4) based on the main effect of sex ($F(1,29)=8.46$, $p<0.05$) without a sex x treatment interaction ($p>0.1$). After 30 minutes of restraint stress, offspring of dams treated with CGP 55845 displayed a blunted stress response as indicated by decreased serum corticosterone levels relative to vehicle treated controls (vehicle = 163.0 +/- 13.1, CGP 55845 =

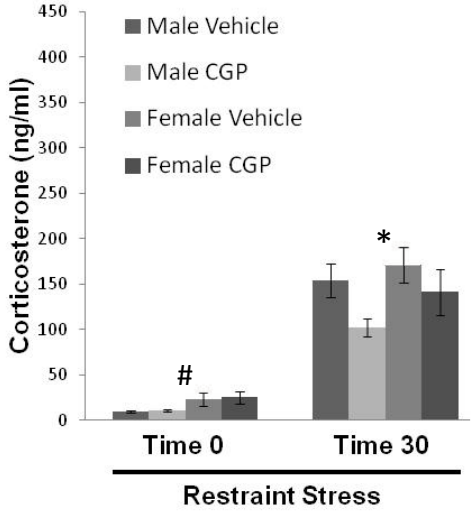

Figure 4.7. Graph depicts corticosterone levels at baseline and following restraint stress. At baseline, females had increased serum corticosterone concentration relative to males (# indicates a statistically significant main effect due to sex $p<0.05$, while the sex by treatment interaction was not significant ($p>0.1$). After 30 minutes of restraint stress, offspring from mothers treated with CGP 55845 had blunted stress responses indicated by a smaller increase in corticosterone concentration relative to vehicle treated controls (* indicates a statistically significant main effect due to treatment $p<0.05$).

121.9 +/- 15.0) based on the significant main effect of treatment ($F(1,31)=4.3$, $p<0.05$) without a sex by treatment interaction ($p>0.1$).

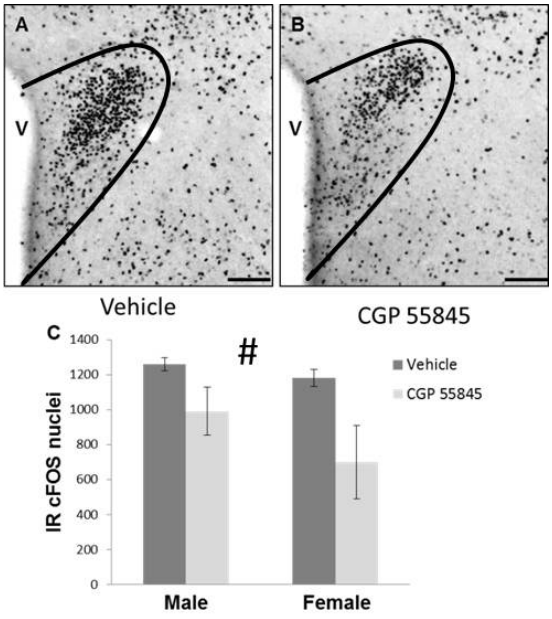

Figure 4.8. Restraint stress-induced FOS expression in and around the PVN. Immunoreactive Fos in the PVN 1 hour after release from restraint, vehicle treated (A) and CGP 55845 treated (B) female mice. Adult offspring of mothers treated with CGP 55845 during pregnancy had a decreased immunoreactive Fos response in the PVN one hour after release from restraint stress in both males and females (90min from start of restraint stress). PVN region where immunoreactive nuclei were counted is outlined in black. Data are Mean +/- SEM. # indicates main effect of treatment, $p<0.05$. V = 3rd ventricle, scale bars represent 200µm.

Discussion

Genetic and environmental influences in the developing fetus contribute to an organism's ability to respond to stressors as an adult and its likelihood of developing stress related pathology (Viveros et al., 2012). Identifying and understanding fetal antecedents to adult psychiatric disorders will enhance prevention and treatment strategies specific to sex, genotype, and known environmental conditions. The results of the current study suggest that 1) GABA acts through $GABA_B$ receptors to restrict cell migration in the PVN, and 2) that embryonic $GABA_B$ receptor antagonism alters the ability to display behaviors in adult mice that are used as models for human psychiatric disorders. Embryonic $GABA_B$ receptor antagonism caused neurons in the PVNs of organotypic slices from females to increase their speed of movement and caused a lateral shift in the location of cells containing ERα from the PVN to the perifornical area in adult females. This shift in cell location corresponded with an increase in anxiety-like behavior and decrease in depressive-like behaviors in female mice. In male offspring, embryonic $GABA_B$ receptor antagonism resulted in a hyperactivity-like phenotype and decreased depressive-like behaviors. These findings suggest that the same neurochemical insult can result in an altered phenotype for a female

predominant disorder in females (anxiety) and an altered phenotype for a male predominant disorder in males (hyperactivity). Importantly, they indicate that the phenotypes seen in mice lacking functional $GABA_B$ receptors (increased anxiety-like and decreased depression-like behaviors) might be explained by altered embryonic brain cellular patterning.

The embryonic PVN is enriched with immunoreactive $GABA_B$ receptor subunits and is surrounded by immunoreactive GABA and GAD (McClellan et al., 2010) leading to the hypothesis of GABA acts through $GABA_B$ receptors to influence PVN development. In the current study, alterations in cell movements visualized directly in organotypic slice cultures further establish a role for GABA in driving cell movements in the developing PVN. The largest effect of $GABA_B$ receptor antagonism appeared to be on cells in slices from female embryos that were found near the boundary of PVN where movement speeds were increased. It is interesting that the sex-dependent effect on neuron movements was seen at E13, a time point prior to robust testosterone production by murine testes. Thus, the observed sex difference was likely due to male (XY) and female (XX) genetic differences rather than hormone-dependent mechanisms (Majdic & Tobet, 2011).

GABA acting through $GABA_B$ receptors restricts cell movements in other regions of the developing hypothalamus (Davis et al., 2002; McClellan et al., 2008). This influence differs from the role of $GABA_B$ receptor activation on neuron migration in the cerebral cortex (Behar et al., 1999, 2000, 2001; Lopez-Bendito et al., 2003), where it has been suggested to stimulate migration. $GABA_B$ receptors couple to second messenger pathways based on pre or postsynaptic location and brain region (Colmers and Williams 1988; Bettler et al., 2004; Gassman and Bettler, 2012) and it is possible that they are coupled differently in developing hypothalamus relative to developing cerebral cortex.

Treating pregnant mice with a $GABA_B$ receptor antagonist for 7 days was sufficient to create a chemoanatomical phenocopy of a $GABA_B$ R1 subunit knockout mouse. This provides evidence consistent with a hypothesis that GABA restricts cell migration for some neurons to remain within the PVN. The consequences of an animal having fewer cells responsive to estrogens in the PVN and more cells responsive to estrogens in the perifornical region are unclear. 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), aggressive behaviors and autonomic cardiovascular responses (Peyron et al., 1998; Steininger et al., 2004). This juxtaparaventricular region has also been shown to be a massive communication hub with projections to or inputs from half of all identified gray matter regions in the entire brain (Hahn and Swanson, 2010). The lateral population of cells containing immunoreactive ERα is comprised of approximately 70% GABAergic neurons with projections to the PVN. This lateral population of ERα containing cells may be part of the limbic inputs that exert an inhibitory tone on the PVN (Herman et al., 2005; Weiser et al., 2008B). They are in the same region as TRH expressing neurons (ERa content unknown), which may be subject to fetal programming influences by synthetic glucocorticoid treatments (Carbone et al., 2012). Interestingly, in the adult rat, estradiol can enhance the sensitivity of PVN CRH neurons in the HPA response to stress (Lund et al., 2006). The reorganization that occurs in female mice as a result of embryonic $GABA_B$ receptor antagonism could change the balance of estrogenic responses in and around the PVN.

Basal and stimulated HPA axis activity has been shown to be higher in females than males (Critchlow et al., 1963; Handa et al., 1994; Rhodes and Rubin, 1999; Iwasaki-Sekino et al., 2009). This corresponds to sex steroid influences on the expression of peptides in the PVN (Haas and George, 1988; Patchev et al., 1995). There are, however, relatively few findings of

sexual dimorphism (chemoarchitectural or anatomical) in the PVN. Here, a sex difference in the location of immunoreactive ERα containing cells was revealed when $GABA_B$ receptors were antagonized selectively during fetal development. This might be directly related to sex differences in the movement characteristics of cells in the fetal PVN region in response to $GABA_B$ antagonism noted above as well as to sex differences in behavioral responses related to anxiety.

The final common pathway for all circuits influencing the HPA axis is the PVN. 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 also has been shown in human studies (Bao et al., 2008; Bao and Swaab, 2007; Wang et al., 2008). In the current study, embryonic pharmacological blockade of the $GABA_B$ receptor resulted in altered cytoarchitecture in the region of the PVN and this altered cytoarchitecture corresponded with altered anxiety- and depression-like behaviors. Unfortunately, a direct cause and effect relationship cannot be drawn as the entire developing embryo was exposed to the CGP compound. There is, however, correlational evidence that the observed alterations in physiology and behavior are related to a CGP 55845 mediated reorganization of the PVN. When the ERα distribution was considered as a ratio of medial to lateral immunoreactivity (first two (medial) columns divided by the immunoreactivity found in the last two (lateral) columns), a medial ERα distribution was strongly correlated with greater corticosterone concentrations after 30 minutes of restraint stress ($r = 0.97$, $p=0.001$). Put another way, mice with more cells containing ERα shifted lateral to the PVN had less robust stress responses. Also, immunoreactive FOS within the PVN was significantly correlated with immobility time in the tail suspension test in all animals tested ($r = 0.60$, $p<0.05$)

and sucrose preference was significantly correlated with corticosterone levels after 30 minutes of restraint in CGP treated animals ($r = 0.51$, $p=0.05$).

Generally, fetal treatment with CGP 55845 resulted in blunted HPA axis activation in adult mice. This was evident in FOS immunoreactivity within the PVN and in corticosterone concentrations after 30 minutes of restraint stress. This may be partially PVN specific as statistical analysis of immunoreactive FOS cell counts in a region dorsal and lateral to the PVN yielded no significant effects or interactions. These indices were treatment-dependent, but not sex-dependent. That is the same relationship seen in the depression-like behavioral tests of tail suspension and sucrose preference where embryonic exposure to CGP 55845 caused decreased depression-like behavior patterns independent of sex.

Despite the blunted corticosterone response in both sexes, females showed increased anxiety-like behavior in the elevated plus maze. This apparent disparity may be related to the timing and relative intensity of stressors in each of the tests. For instance the stress response was measured with corticosterone concentrations after 30 minutes of restraint stress (similar longer term measurement for FOS), while the elevated plus maze was conducted over 5 minutes and did not involve an acute stressor other than minor handling and the presence of open and elevated platforms. These results could be considered paradoxical when viewed from the standpoint of anxiety contributing to and being comorbid with depression. Nonetheless, these results of increased anxiety-like behavior but decreased depression-like behavior replicated the phenotypes seen in $GABA_B$ receptor knockout mice (Mombereau et al., 2004; Gassmann et al., 2004).

In summary, the results of the current study suggest that GABA acts through the $GABA_B$ receptors to direct cell movements in the developing female PVN with potential long-term

consequences in vivo. Female offspring of dams treated with the $GABA_B$ receptor antagonist, CGP 55845 had altered PVN cytoarchitecture and in the neighboring perifornical area that was associated with altered anxiety-like behavior. Independent of sex, embryonic $GABA_B$ receptor blockade caused decreases in depression-like behavior, and an attenuated adult corticosterone response to restraint stress concomitant with decreased neuron activation (FOS induction) in the PVN.

CHAPTER 5. DISCUSSION AND FUTURE DIRECTIONS

This work collectively demonstrates that GABA acts through both the $GABA_A$ and $GABA_B$ receptor to direct development of the PVN.

Pharmacological blockade of the $GABA_A$ receptor resulted in fewer cells containing immunoreactive ERα in the PVN and the perifornical region. This result could have been mediated by either decreased proliferation of progenitors giving rise to ERα expressing neurons or down regulation of ERα gene expression, translation or protein stability. Although, as the pharmacological treatment ended days before tissue collection, effects on translation and protein stability are unlikely. GABA has been shown to stimulate proliferation (related to embryonic Cl^- gradient and GABA induced depolarization) during development and regulate gene expression. Thus it is likely that the observed effect of $GABA_A$ signaling on PVN development is mediated by regulating cell proliferation and/or gene expression. However, as there was evidence that signaling through the $GABA_B$ receptor might be important for influencing cell placement *in vivo*, the remaining dissertation focused on $GABA_B$ receptor signaling.

Using a line of mice that lack functional $GABA_B$ receptors, GABA influence on PVN development was investigated. While assaying $GABA_B$ receptor-mediated migration events, several interesting observations were made. First, at birth CRH is actually present in the posterior pituitary gland. While this was previously mentioned in a paper characterizing the first antibody for CRH, no other reports or implications of centrally produced and regulated CRH being present in general circulation at birth have been found. Dr. Vale was famous for, amongst many other things, insisting that CRH should be called corticotropin releasing factor instead of

hormone. This was because, for something to be called a hormone it should be found in general circulation. The current work may have been able to persuade Dr. Vale that CRH is at least expressed in a position (posterior pituitary) to allow it to function as a hormone in general circulation, granted during a narrow perinatal time frame.

There was also evidence for an alternate site of ontogeny for CRH neurons in the PVN. Identifying this possible alternate site of CRH neuron neurogenesis and alternate migratory route may be important for two reasons. First, neurons that arise from different locations and different progenitor pools likely have a different epigenetic program, creating the possibility for alternate regulation of CRH expression and release. Similarly, if CRH neurons migrate through unique regions with different microdomains and associated chemical signals on their way to the PVN, this also creates the possibility for alternate epigenetic programming and CRH regulation. Finally, a sex- and region-specific influence of $GABA_B$ receptors on CRH peptide content in the PVN was identified in $GABA_B$ R1 knockout mice. Increased amounts of CRH mRNA, CRH peptide and CRH containing cells have been associated with human depression (Raadsheer et al., 1994,1995). When examining the downstream effects of long term HPA axis activity it is interesting to note that disorders resulting from dysregulation of the HPA axis, such as major depressive disorder and anxiety related disorders, are more prevalent in females than males. Our data that females but not males lacking functional $GABA_B$ receptors have more CRH peptide in the PVN might help to explain some of the predisposition of disorders resulting from HPA axis dysregulation in females.

To build upon the developmental findings in neonatal mice lacking functional $GABA_B$ receptors and to investigate the portion of behavioral deficits in GABA receptor deficient mice that can be explained by altered development, $GABA_B$ receptors were antagonized during embryonic development. Acute and female-specific effects of $GABA_B$ receptor blockade were seen in *ex*

vivo cell movements in the developing PVN. These acute *ex vivo* effects mirrored female specific changes in cell placement in the adult PVN of animals that received embryonic $GABA_B$ receptor antagonism. Embryonic $GABA_B$ receptor antagonism also resulted in sex-specific changes in activity and anxiety-like behaviors. Finally, in both sexes, embryonic $GABA_B$ receptor antagonism caused decreased stress response and decreased depression-like behaviors in adult mice. These results almost completely recreate the phenotypes seen in the $GABA_B$ receptor knockout mice.

This work helps to highlight/describe 1) the highly organized structure of the PVN (not only in arrangement of adult cell type but also in mechanisms of development), 2) sex-specific nature of developmental mechanisms of the brain (PVN), and 3) the importance of understanding the developmental influence of genetic mutations when analyzing complex systems or behaviors. This work provides robust support for GABA influencing cell migration and other developmental processes in the PVN and hypothalamus via the $GABA_B$ receptor.

OPEN RESEARCH QUESTIONS/FUTURE DIRECTIONS:

Sex-Specific Nature of Findings:

Why the developing female PVN is more dependent on $GABA_B$ signaling than that of the male remains an open question. The simplest explanation would be that there are sex differences in the GABA concentration seen by the developing PVN or in the distribution of $GABA_B$ receptors in the developing PVN. A preliminary survey of $GABA_B$ R1 subunit immunoreactivity in embryonic mice suggested no difference in $GABA_B$ R1 protein expression between the sexes in the PVN (Stratton and Tobet unpublished observations). While sex differences in concentrations of GABA have been reported, the studies have either been in adult or early

postnatal animals, and PVN was contained in tissue blocks with other hypothalamic nuclei (Flugge et al., 1986; Frankfurt et al., 1984). Kelly and colleagues have shown the estradiol and some other sex steroid metabolites can attenuate $GABA_B$ receptor activation with baclofen through membrane estrogen receptors (Qiu et al., 2008). This might create a scenario where for instance the $GABA_B$ receptor was already antagonized in the male. Alternatively a class of receptor modifying auxiliary subunits has recently been described for the $GABA_B$ receptor and sex differences may exist in these subunits.

Alternate Migratory Pathways:

Some of our findings (Chapters 3 and 4) suggest that some cells of the PVN arise from regions other than the proliferative zone directly adjacent to the developing PVN. These findings include the presence of CRH immunoreactivity first being found lateral to the hypothalamus and being found later on during development spread between the PVN and non hypothalamic regions (Chapter 3). Also, in fetal organotypic brain slices, robust migration in the dorsal/ventral axis and movements in the lateral to medial direction were observed. If this migration brings cells to the PVN with alternate expression patterns (e.g. proteins regulating CRH release) the field would greatly benefit from learning the site of origin of these neurons. One way to determine this would be by using reporter mice in which the expression of a fluorescent protein was under the control of the promoter from a PVN-specific gene product like AVP (Moritoh et al., 2011) or CRH (Alon, 2009). Personal communication with investigators who currently have CRH reporter mice have been insufficient to determine fidelity of fluorescent protein expression or at what time point in development expression is robust enough to track cell movements in fluorescent video microscopy. It would behoove someone to actually commit the resources to determine if these transgenic mouse lines could be feasible models for study of PVN development.

Another approach for which we have done some pilot work, involves fluorescently labeling neurons based on DNA synthesis and tracking individual cells that were in S-phase of the cell cycle while exposed (either in cell culture bath or *in vivo*) to a specific DNA base analog. We have been able to determine that cells will take up the modified base, 5-azido-2' deoxyuridine, and incorporate it into the DNA and that this modified base will under go "CLICK" cycloaddition reaction with fluorescently labeled alkynes. This is a novel method to birth date label cells. However, when taken to the next step, which relies on a cell permeable fluorescent molecule attached to a cyclooctyne structure, which allows for spontaneous cycloaddition with the azide modified base, the resultant fluorescent signal is inappropriate and does not correspond with existing birthdate labeling strategies done in parallel. Further chemical optimization is required to direct the fluorescent cyclooctyne to the nucleus. More detailed description of this work is contained in the appendix.

Region specific and time specific lack of $GABA_B$ receptor signaling:

Work presented in chapter 4 was aimed at dissecting the contribution of developmental $GABA_B$ signaling from lifelong $GABA_B$ signaling (knockout mice) on stress-related behaviors in mice. While we were successful in this aim, our results include the contribution of altered development in the entire embryo as antagonist treatment was through I.P. injection to the dam. In order to causally link altered PVN cytoarchitecture with altered behavior both temporal (embryonic) and regional (PVN) control of receptor signaling must be obtained. Although outside of the current resources of the lab, it would be possible to construct a genetically modified mouse for which the $GABA_B$ receptor gene was interrupted selectively in the developing PVN then allowed to re-express after birth. A mouse with critical regions of the $GABA_B$ R1 subunit gene floxed does exist (Gangadharan et al., 2009). PVN selective promoter (Sim1) driven CRE mice also exist (Fyffe et al., 2008). Others have used similarly modified mice with the addition of drug inducible

(tamoxafin) expression of recombinases to add back gene function at a given time point post development (Hayashi and McMahon et al., 2002).

Negative results and abandoned projects:

Not all of our work over the past four years has been incorporated into the above chapters, nor has it all been published. Most of this work falls into the category of negative results, which are inherently difficult to interpret. Some projects also fall into the categories of scooped ideas and harsh cost benefit analysis. While not detailing all of my swings and misses over 4 years, below are brief summaries of projects that ended up not leading to publication in hopes that others will either pick up the projects or avoid them as appropriate.

As BDNF has been widely implicated in depression and is expressed in the developing PVN (McClellan et al., 2010) we obtained three lines of genetically modified mice to determine BDNF's role in PVN development and stress related behaviors. Our first attempt involved breeding mice that had BDNF exon 5 floxed (necessary for all known BDNF expression) with mice expressing the CRE recombinase under the control of the SIM1 promoter (a transcription factor necessary for PVN development). Upon immunohistochemical analysis of BDNF in the PVN, there was no difference in immunoreactivity between Sim1 CRE mice and Sim1 CRE mice that had both alleles of BDNF exon 5 floxed. This could be interpreted as 1) CRE recombinase not being able to remove BDNF exon 5 from the genome or 2) BDNF positive cells in the PVN coming from a non-SIM1 expressing progenitor pool. Attempting to determine which possibility was true did not appear to be fruitful or worthwhile avenues of research based on lab resources. The only phenotype seen with these mice was a dramatic increase in body weight which was present in the floxed mice (not yet crossed with the recombinase containing mice). Somehow, the additional genetic information inserted around exon 5 altered gene function enough to make

these mice obese. We then obtained the global BDNF knockout mouse from Jackson Labs. Immunohistochemical analysis of CRH, nNOS, AVP, and NPY showed no detectible difference in PVN cytoarchitecture between knockout and wild type mice.

While the rat PVN is very well described in the literature, no detailed map of the mouse PVN was available. This is partly due to the fact the magnocellular and parvocellular components of the rat PVN are well separated, while in the mouse magnocellular and parvocellular neurons are intermingled with unclear boundaries. We wanted to 1) provide a detailed map of PVN adult cytoarchitecture and 2) determine which neurons projected centrally to control autonomic function and which neurons projected to the median eminence or posterior pituitary to regulate endocrine functions. We began labeling endocrine neurons based on Evans Blue uptake. Neurons projecting to the median eminence or posterior pituitary have their axon terminals outside of the blood brain barrier and will internalize dies in circulation. We also conducted track tracing experiments by placing fluorescent lipophilic dies in specific brain stem regions in hopes of identifying the cell bodies of neurons in the PVN that regulated autonomic function. While optimizing these techniques a paper was published that did exactly what we were trying with slightly different methods (Biag et al., 2012). The project was shelved and we wrote a Faculty of 1000 review of their paper.

As described above, we hypothesized that some cells of the PVN originate from regions other than the proliferative zone adjacent to the PVN as is currently assumed. To prove this, we began removing regions from fetal organotypic brain slices and letting them age *in vitro*. If there were no neurons of a certain type present in the PVN after the removal of a specific brain region, that would provide evidence for where they were migrating from. Unfortunately, at this time, the breeding colony was suffering from cannibalism and lack of fertility and other funded

experiments were in jeopardy. While a promising approach, other efforts were more fruitful and further investigation along these lines was not attempted.

All of this dissertation work was aimed at determining mechanisms of hypothalamic development and investigating the impact of altered development on adult hypothalamic function or creating advanced tools to assist in the above investigations. While some of the efforts listed did not yield significant advancement, this work collectively demonstrates that GABA acts through both the $GABA_A$ and $GABA_B$ receptor to direct development of the PVN.

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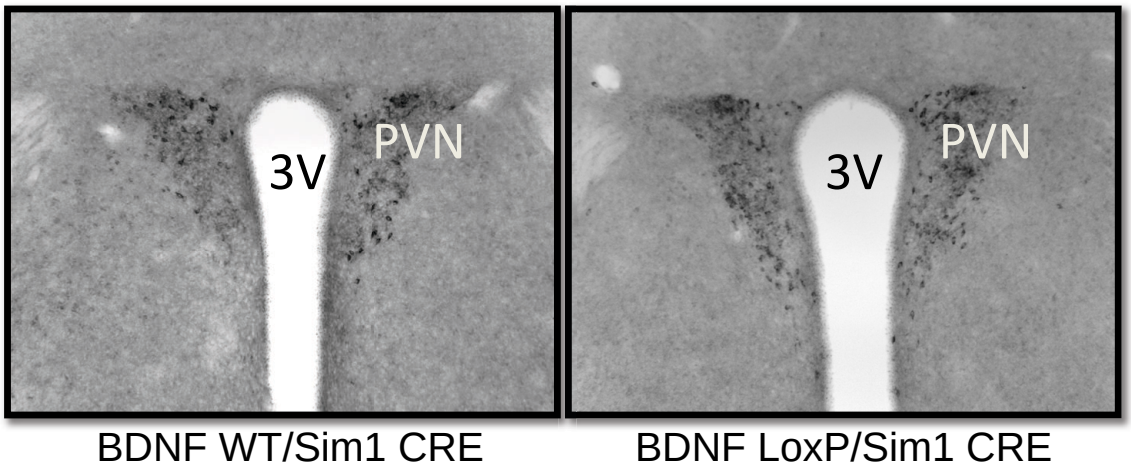
APPENDIX

Sim1 Cre/BDNF LoxP mouse phenotype:

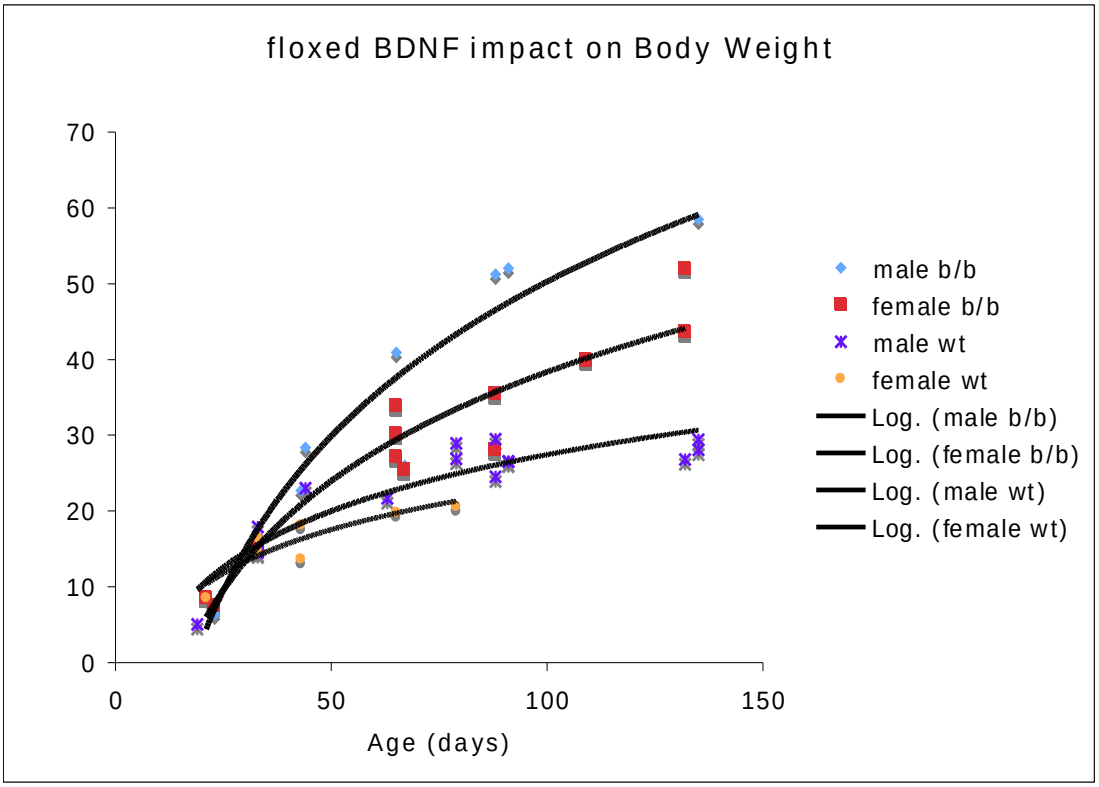
As brain derived neurotrophic factor (BDNF) has been identified as both a neurotrophic factor during development and an important molecule in both anxiety and depression we took steps to test hypotheses regarding BDNF's role in PVN development. This work involved targeted disruption of the BDNF gene in hypothalamic regions that express the transcription factor Sim1 during development. Cre recombinase was expressed under the control of the Sim1 promoter. Theoretically, the recombinase should catalyze recombination and removal of genetic information at LoxP sites flanking exon 5 of the BDNF gene. This exon is thought to be necessary for all BDNF activity. Mice heterozygous for the BDNF LoxP allele that also carried the Sim1Cre allele were bread together. Neonatal and adolescent offspring were perfusion fixed and genotypes were assessed via PCR.

Offspring homozygous for the BDNF LoxP allele that also carried the Sim1Cre modification showed no difference in the amount of immunoreactive BDNF in the PVN compared to mice that only carried the Sim1Cre modification (Appendix Figure 1). Other immunoreactive proteins in the PVN were also analyzed and again there were no differences were noted (data not shown). There was, however, one striking phenotype seen in animals that were homozygous for the BDNF LoxP allele without the presence of Sim1Cre modification compared to wild type animals. Animals with two copies of the BDNF LoxP allele developed an obesity phenotype (Appendix Figure 2). It is likely that the insertion of the LoxP genetic sequence disrupted untranslated 5' mRNA sequence that regulates mRNA trafficking.

It is our interpretation of these negative results is that either 1) the CRE recombinase under the control of the Sim1 promoter was not able to catalyze the homologous recombination and removal of BDNF exon 5 or 2) the BDNF immunoreactive neurons in the PVN arise from non Sim1 expressing progenitor pools.



Appendix Figure A1. No difference in immunoreactive BDNF in wild type mice that carry the Sim1 CRE modification (left) and mice homozygous for BDNF LoxP that carry the Sim1 CRE modification (right).



Appendix Figure A2. Both males and females homozygous for BDNF LoxP allele (b/b, floxed) weigh as much as two times more than wild type (wt) mice. (Y axis = grams)

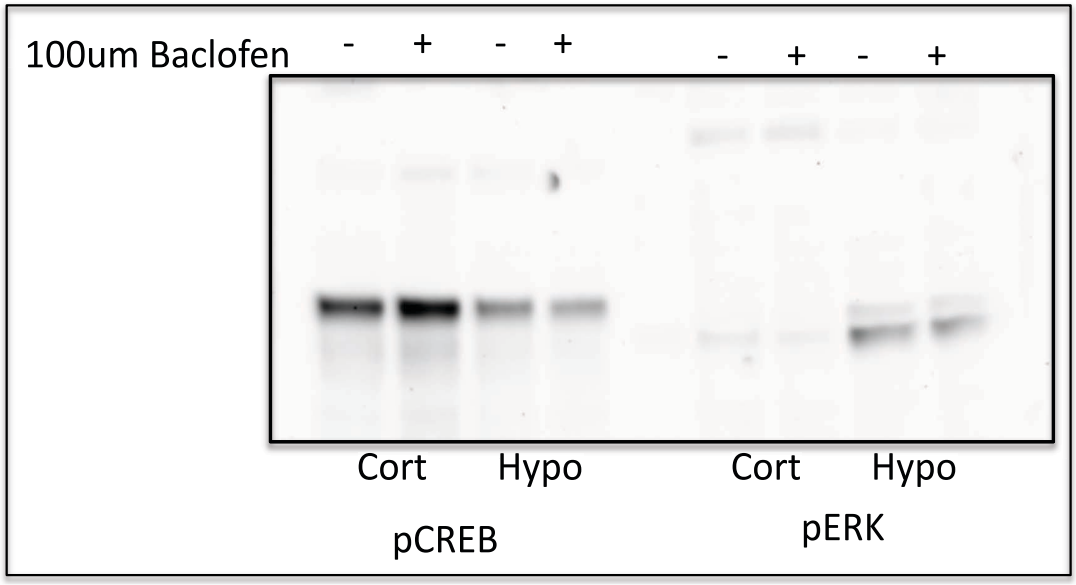
Global BDNF knockout mouse PVN phenotype:

Because of the inability to use the SIM1 Cre/BDNF LoxP genetic approach to test hypotheses, we acquired the global BDNF knockout mouse. These mice do not make functional BDNF peptide at any time, anywhere in the entire animal. Mice homozygous for the BDNF knockout allele died within the first postnatal week in our hands (others reported survival to postnatal day 14). Heterozygous mice were mated and neonatal offspring were perfusion fixed and immunohistochemical investigation was carried out for proteins commonly found in the PVN including NPY, AVP, OT and CRH. No changes in protein location or amount were noted.

These results indicate that BDNF does not play an active role in embryonic development of the PVN or at least there is a redundant mechanism that takes over in the absence of BDNF.

$GABA_B$ receptor activation and embryonic CREB phosphorylation:

Our work in the hypothalamus indicates that GABA acts through the $GABA_B$ receptor to decrease the rate of neuron migration. In the cortex however, GABA acts through the $GABA_B$ receptor to increase the rate of neuron migration. Thus it appears that the $GABA_B$ receptor is alternatively coupled to second messenger molecules in cortex compared to the hypothalamus during embryonic development. One pathway that has been shown to be influenced by $GABA_B$ receptor signaling is cAMP dependent protein kinase A (PKA). PKA then phosphorylates CREB and regulates gene expression. Organotypic brain slices from mouse embryos were treated with the $GABA_B$ receptor agonist baclofen. Then, areas of cortex and hypothalamus were harvested separately and snap frozen. Later, tissue was homogenized in RIPA buffer with protease and phosphatase inhibitors and processed for western blot that was probed with anti phospho-CREB primary antibody. In the cortex samples, CREB phosphorylation was robustly increased. Alternately, in the hypothalamic samples, CREB phosphorylation was slightly decreased (Appendix Figure A3). This indicates that $GABA_B$ receptors are indeed alternatively coupled to downstream pathways in cortex and hypothalamus during embryonic development.



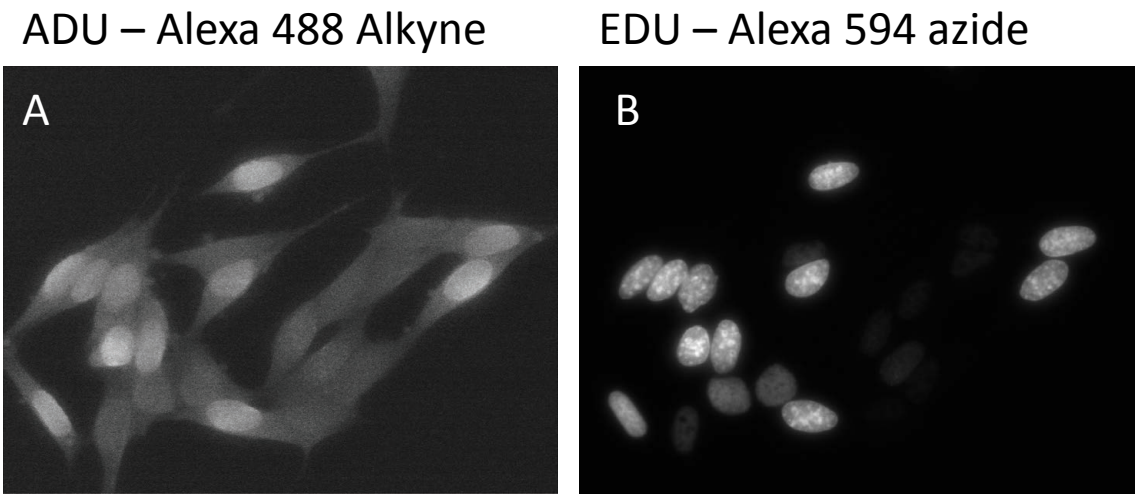
Appendix Figure A3. Induction of CREB phosphorylation by Baclofen treatment in cortex but not hypothalamus.

Copper free CLICK chemistry based method to label DNA synthesis:

The formation of functional cell groups in the hypothalamus during development is critical for many physiological functions such as reproduction, stress response, blood pressure regulation, temperature regulation, feeding and the regulation of the autonomic nervous system. The current model of hypothalamic development includes and inside out model of radial migration in which the earliest born (last mitotic event) neurons migrate the farthest from the proliferative ventricular zone toward the pia surface. There is an assumption built into this model that some non-radial migration occurs as there are clear voids is specific regions of the hypothalamus in neuron birthdate labeling experiments. We hypothesize that there is not only abundant non-radial migration but also infiltration of neurons that originate from sites outside of the hypothalamus. This hypothesis is supported by our observations of cells moving toward the ventricle of the hypothalamus from lateral regions in fetal organotypic slice video microscopy

and the above mentioned voids in neuron birthdate studies. Direct testing of this hypothesis has been limited by 1) latency of fluorescent protein expression in specific reporter lines of mice and 2) incompatibility of current neuron birthdate labeling and visualization strategies with living tissue. We have taken steps to develop a novel approach to label mitotic events that is compatible with living tissue and will make direct testing of our hypothesis feasible. As cells progress through the cell cycle, DNA is doubled during S-phase. If a modified DNA base, nucleoside, is close enough in chemical structure to the endogenous base and present during S-phase, the cell will incorporate this modified base into its genomic DNA. Subsequent detection of the modified base, either through immunohistochemistry (BrDU), radiography (tritiated thymidine), or chemistry (EdU) has been used for labeling the cells that progressed through S phase at a given time point. Unfortunately, these methods have not been amenable to viewing these cells in living tissue as steps in the visualization process are incompatible with cell survival. Using existing chemistry in a novel application we have developed a method to label and visualize cells that have incorporated a modified DNA base into their DNA in living cells. Briefly, the modified base, 5-azido-2'-deoxyuridine (ADU), is incorporated into the DNA during S-phase and fluorescently detected via a cell permeable cyclooctyne linked to a fluorescent coumarin molecule. Copper free CLICK chemistry, using cyclo-octynes allows catalyst free covalent bonding to occur between the carbon - carbon triple bond in the Cyclo-octyne and the N_3 group in the ADU.

Thus far, we have been able to demonstrate that ADU is incorporated into newly synthesized DNA and can be detected with fluorescent alkynes using standard copper dependent CLICK chemistry in fixed cell. Unfortunately, the coumarin coupled cyclo-octyne, which should spontaneously react (via strain induced cyclo addition) with the azido group on ADU, does not yield an appropriate fluorescence pattern (not nuclear and does not overlap with cells co-treated with EDU).



Appendix Figure A4. ADU and EDU detection in cells that were cotreated with ADU and EDU. Cells were then sequentially labeled with Alexa 488 alkyne aor alexa 594 azide.