Serotonin 1A Receptor Signaling in the Hypothalamic Paraventricular Nucleus of the Peripubertal Rat

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LOYOLA UNIVERSITY CHICAGO

SEROTONIN 1A RECEPTOR SIGNALING IN THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS OF THE PERIPUBERTAL RAT

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN NEUROSCIENCE

BY
MAUREEN LYNN PETRUNICH RUTHERFORD
CHICAGO, ILLINOIS
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For my husband, Billy
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine, serotonin</td>
</tr>
<tr>
<td>5-HT$_{1A}$</td>
<td>serotonin 1A receptor</td>
</tr>
<tr>
<td>(+)8-OH-DPAT</td>
<td>(+)8-hydroxy-2-(di-n-propylamino)-tetralin hydrobromide, a selective 5-HT$_{1A}$ receptor agonist</td>
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<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>Akt</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotrophin releasing hormone</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FLX</td>
<td>fluoxetine, a commonly used SSRI</td>
</tr>
<tr>
<td>G protein</td>
<td>guanine nucleotide regulatory protein</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin, kinase responsible for phosphorylating Akt at Ser473</td>
</tr>
<tr>
<td>pAkt</td>
<td>phosphorylated (activated) protein kinase B</td>
</tr>
<tr>
<td>PD</td>
<td>postnatal day</td>
</tr>
<tr>
<td>pERK</td>
<td>phosphorylated (activated) extracellular signal-related kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase, an upstream activator of Akt</td>
</tr>
<tr>
<td>PDK1</td>
<td>phosphoinositide dependent kinase 1, kinase responsible for phosphorylating Akt at Thr308</td>
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<tr>
<td>PTX</td>
<td>pertussis toxin, a toxin produced by the bacterium <em>Bordetella pertussis</em></td>
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<tr>
<td>PVN</td>
<td>paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SSRI</td>
<td>selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>WAY100635</td>
<td>(N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinyl)cyclohexane-carboxamide, a selective 5-HT$_{1A}$ receptor antagonist</td>
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Serotonin (5-HT) is a ubiquitous neurotransmitter in the brain that is involved in various physiologic functions including the regulation of hypothalamic hormones and has been implicated in various mood disorders such as depression. Preclinical and clinical data from studies in adults have shown that antidepressant drugs produce time-dependent changes in serotonergic and other systems and can also normalize dysfunction associated with the hypothalamic-pituitary-adrenal (HPA) axis. To date, our understanding of the mechanisms of 5-HT receptor signaling and the actions of drugs on serotonergic function have been derived from extensive preclinical research carried out using cell lines in vitro or in adult animal models in vivo. Fewer studies have investigated serotonergic signaling mechanisms or the effects of antidepressants (i.e., selective serotonin reuptake inhibitors (SSRIs)) in animal models prior to sexual maturation. This is a scientific and clinically relevant issue as (1) SSRIs, the most effective pharmacological option for treating mood disorders in children and adolescents, are being increasingly prescribed and may be associated with suicidal thoughts or behaviors in these age groups and (2) preclinical studies in rodents indicate that SSRI-induced modulation of the serotonergic system prior
to sexual maturation produces effects that are distinct and more long-lasting than those produced in adults. To date, few if any studies have investigated mechanisms of 5-HT receptor signal transduction in peripubertal hypothalamic neurons \textit{in vivo} and their regulation by SSRIs, despite a wealth of existing comparative data on serotonin 1A (5-HT\textsubscript{1A}) receptor signaling in adult hypothalamic rat paraventricular nucleus (PVN).

Given the clinical relevance of potential age-dependent differences in serotonergic signaling and regulation of hypothalamic function, the objective of the studies in this dissertation was to identify and characterize the mechanisms of 5-HT\textsubscript{1A} receptor signaling in the peripubertal hypothalamic PVN.

The data generated by studies in this dissertation project provide the first \textit{in vivo} evidence that 5-HT\textsubscript{1A} receptors in the peripubertal PVN can activate multiple responses: (1) oxytocin and adrenocorticotropic hormone (ACTH) plasma hormone responses, (2) activation of extracellular signal-regulated kinase (ERK), and (3) activation of protein kinase B (Akt). The data also demonstrate that these pathways may be differentially responsive to different classes of 5-HT\textsubscript{1A} receptor agonists. (+)8-OH-DPAT (an aminotetralin), acted as a “full” agonist on each of the respective pathways, while tandospirone (an azapirone) exhibited “partial” agonist activity on activation of Akt but exhibited “full” agonist activity on neuroendocrine responses and activation of ERK. 5-HT\textsubscript{1A} receptors produce a rapid and prolonged activation of ERK in the peripubertal
PVN, unlike the rapid but more transient response in the adult PVN. In addition, the 5-HT$_{1A}$ activation of ERK may be expressed only in certain populations of neuroendocrine cells in the peripubertal PVN. These studies have also revealed some unique aspects that G$\alpha$ proteins in the peripubertal PVN, since: (1) there are age-dependent increases only in G$\alpha_o$ levels, (2) G$\alpha_i3$ and G$\alpha_o$ are not reduced by intra-PVN injection with pertussis toxin, and (3) G$\alpha_z$ proteins are not reduced by 7 or 14 days of fluoxetine treatment. We also have determined that chronic fluoxetine treatment desensitizes 5-HT$_{1A}$ receptor-mediated phosphorylation of GSK3$\beta$ in the absence of changes in its canonical upstream kinase, Akt. These data suggest fluoxetine may induce changes in phosphatase activity or that there may be a different upstream kinase mediating the phosphorylation of GSK3$\beta$ in the peripubertal PVN.

In conclusion, the present studies determined the mechanisms of 5-HT$_{1A}$ receptor-mediated signaling in peripubertal PVN and identified some aspects of 5-HT$_{1A}$ receptor signaling that differ from those previously identified in adults. These findings may be clinically relevant with respect to facilitating a better understanding of mechanisms mediating the therapeutic and/or side effects of SSRIs prescribed to young patients or to identify novel drug targets to treat mood disorders in children and adolescents.
CHAPTER 1

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is primarily found in the gastrointestinal tract, platelets, and central nervous system, where it acts as a monoamine neurotransmitter. In the brain, 5-HT can function as a “developmental signal” to direct biochemical and morphological differentiation of 5-HT-containing cells localized in the raphe nuclei (Lauder 1990; Rubenstein 1998). The raphe nuclei are discrete groups of cells located in the brainstem that contain perikarya of serotonergic neurons that constitute the ascending and descending serotonergic projections to almost every area of the central nervous system. Two prominent ascending projections that innervate various brain regions (including the hypothalamus) arise from neurons located in the dorsal and median raphe nuclei.

5-HT plays a prominent role in the regulation of behavior, mood, appetite, perception, circadian rhythms, body temperature, and cognitive functions such as memory and learning (Berger et al., 2009). Many studies have linked dysfunction of the serotonergic system with psychological disorders such as depression and anxiety, and many classes of psychotropic drugs used to treat these disorders alter serotonergic signaling.
To date, seven families (5-HT<sub>1-7</sub>) of receptors encompassing a total of 15 subtypes have been identified that mediate the various effects of 5-HT. The serotonin 1A (5-HT<sub>1A</sub>) receptor was one of the first 5-HT receptor subtypes to be identified and cloned (Albert et al., 1990), and has been extensively investigated with respect to its pharmacology and regional distribution in brain. 5-HT<sub>1A</sub> receptors on serotonergic neurons are localized to the soma and dendrites and function as inhibitory autoreceptors to regulate cell firing. This population of somatodendritic autoreceptors has generated a great deal of attention since the desensitization of these receptors (i.e., an attenuation of a receptor-mediated response despite the presence of agonist) plays a prominent role in the therapeutic efficacy of various drugs used to treat mood disorders. 5-HT<sub>1A</sub> receptors also are localized post-synaptically on non-serotonergic cells in various brain regions such as the hippocampus, frontal cortex, and hypothalamus, where they can initiate a variety of signal transduction cascades (Kia et al., 1996a; Kia et al., 1996b; Blier and Ward 2003; Marvin et al., 2010). 5-HT<sub>1A</sub> receptors in the hypothalamus are involved in the serotonergic stimulation of various hormones and regulation of the hypothalamic-pituitary-adrenal (HPA) axis, the interface between the brain and body in response to stress (reviewed in Sullivan-Hanley and Van de Kar, 2003 and Carrasco and Van de Kar et al., 2003). Dysregulation of the HPA axis has been associated with mood disorders, and normalization of
the HPA axis has been suggested to contribute to some of the therapeutic actions of antidepressants (Nemeroff 1996; Holsboer 1999, 2000).

Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine (Prozac®) have completely revolutionized the field of adult psychiatry since they are comparable in effectiveness to tricyclic antidepressant (TCA) and monoamine oxidase inhibitor (MAOI) drug classes in treating several mood disorders but exhibit a more favorable side effect profile than the TCAs or MAOIs (Hirschfeld 1999; Papakostas and Fava, 2006). It is noteworthy that SSRIs, but not tricyclic antidepressants or MAOIs (Ryan 2003; Bostic et al., 2005), have been shown to be effective in treating childhood and adolescent clinical disorders such as depression, obsessive-compulsive disorders, eating disorders, Tourette’s syndrome, autism, and panic disorder (Cohen 2007; Rushton and Whitmire, 2001; Wong et al., 2004). Although SSRIs remain one of the only pharmacological options for treating childhood and adolescent mood disorders, their use has been controversial due to a reported increase in suicidal thoughts or behavior (USFDA, 2004). In the United Kingdom, most SSRI use in children and adolescents has been banned (European Medicines Agency, 2004), while in the United States, the Food and Drug Administration has issued a ‘black box’ warning for SSRI use in children and adolescents. However, in the years since these restrictions were enacted, suicide among child and adolescent age groups significantly increased, indicating an inverse relationship between SSRI prescription rates and suicide.
(Gibbons et al., 2007). Therefore, although the use of SSRIs in children and adolescents may pose an increased risk of suicidal thoughts or behavior in some individuals (Reeves and Ladner 2010), it is likely that these drugs will continue to be prescribed with caution to children and adolescents. These clinical findings suggest that some aspects of SSRI effects on the immature brain may be different than those demonstrated in the adult brain.

Preclinical studies indicate that SSRI-induced modulation of the serotonergic system prior to sexual maturation in rodents produces effects that are distinct and more long-lasting than those produced in adults. For example, in contrast to adult treatment, administration of SSRIs to young animals results in: (1) decreased levels of the serotonin (5-HT) transporter and tryptophan hydroxylase that persist into adulthood (Maciag et al., 2006), (2) increased anxiety-like behavior that persists into adulthood (Ansorge et al., 2004), (3) altered aggressive behavior around the time of sexual maturation (Taravosh-Lahn et al., 2006) and (4) paradoxical anxiogenic responses in several behavioral tests (Oh et al., 2009). These studies demonstrating differences in the way that antidepressants induce neuroadaptations and behavioral effects in the developing brain suggest inherent differences in the mechanisms of serotonergic signaling in the immature vs. adult brain.

The objective of this dissertation is to identify the mechanisms of 5-HT_{1A} receptor signaling and regulation in the peripubertal brain in order to discern
any aspects of 5-HT$_{1A}$ receptor signaling that may be unique to this age relative to mechanisms operative in the adult brain. Several studies in the adult brain indicate that the time necessary for clinical efficacy of antidepressants (~2-3 weeks) corresponds to the time necessary to desensitize 5-HT$_{1A}$ autoreceptors on serotonergic neurons (Le Poul et al., 2000; Czachura and Rasmussen 2000). Desensitization of post-synaptic 5-HT$_{1A}$ receptors on non-serotonergic neurons, also a time-dependent response observed with chronic antidepressant treatment, may contribute to the long-term therapeutic effects of anxiolytic and antidepressant drugs (Borsini 1994). However, the majority of studies investigating the mechanisms of 5-HT$_{1A}$ receptor signaling and desensitization have been primarily conducted either in cell cultures or in vivo in the adult brain. These studies have revealed that the 5-HT$_{1A}$ receptor has the capacity to couple to a variety of signal transduction pathways (e.g., inhibition of adenylyl cyclase, activation of potassium channels, and activation of phospholipase C), with the specificity of 5-HT$_{1A}$ receptor responses dependent on various factors such as cell type, brain region, availability of second messengers, and/or intracellular milieu (reviewed in Barnes and Sharp 1999; Raymond et al., 2001; Filip and Bader 2009; Polter and Li 2010). A number of recent reports have demonstrated post-synaptic 5-HT$_{1A}$ receptors in vivo can also activate mitogenic signaling pathways, including activation of the mitogen-activated protein kinase (MAPK) pathway
(Chen et al., 2002; Sullivan et al., 2005; Crane et al., 2007; Buritova et al., 2009) and the protein kinase B (Akt) pathway (Polter et al., 2010).

In the adult rat hypothalamic paraventricular nucleus (PVN), 5-HT$_{1A}$ receptors can activate independent signaling pathways, utilizing different subsets of $G_\alpha$ proteins as shown in figure 1. Pertussis toxin-insensitive $G_\alpha_z$ proteins mediate the 5-HT$_{1A}$ receptor-induced increase in plasma levels of oxytocin and adrenocorticotropic hormone (ACTH) (Serres et al., 2000a), whereas pertussis toxin-sensitive $G_\alpha_i/o$ proteins mediate 5-HT$_{1A}$ receptor-induced activation of extracellular signal-regulated kinase (ERK) (Crane et al., 2007; Garcia et al., 2006; Sullivan et al., 2005). These responses to acute 5-HT$_{1A}$ receptor activation are independent, since complete blockade of ERK signaling does not alter 5-HT$_{1A}$ signaling of hormone responses (Jia et al., 2007). These respective 5-HT$_{1A}$ receptor-mediated signaling pathways are also differentially responsive to desensitization by SSRIs. Chronic treatment with SSRIs decreases hypothalamic levels of $G_\alpha z$ proteins and desensitizes 5-HT$_{1A}$ receptor-mediated oxytocin and ACTH hormone responses (Raap et al., 1999) but does not desensitize the 5-HT$_{1A}$ receptor-$G_\alpha i/o$-mediated increase in activated (phosphorylated) ERK (pERK) in the PVN (Jia et al., 2006). However, pharmacological blockade of the ERK signaling pathway, either by inactivation of $G_\alpha i/o$ proteins or via blockade of MAPK kinase (the activating kinase for ERK) during SSRI treatment abolishes both the SSRI-induced desensitization of 5-HT$_{1A}$
receptor signaling of hormone responses from oxytocin and corticotrophin releasing hormone (CRH)-containing neurons and the reductions in associated Gαz proteins (Jia et al., 2007). These recent novel findings suggesting the requirement of MAPK in the desensitization of 5-HT1A receptor-mediated hormone responses highlight the complexity of 5-HT1A receptor signaling in adult hypothalamic PVN, and indicate the important role of kinases in depression and the effects of antidepressant drugs.

Multiple lines of evidence have implicated protein kinases/phosphatases in the pathology of mood disorders and the effects of antidepressants. As previously mentioned, intact ERK signaling is required for SSRI-induced desensitization of 5-HT1A receptor-mediated neuroendocrine responses (Jia et al., 2007). Furthermore, in the adult rodent brain, ERK and its regulators have also been shown to be involved with stress-induced behavioral responses (Gourley et al., 2008) and antidepressant-induced amelioration of depressive behavior (Duman et al., 2007; Duric et al., 2010). Another kinase pathway, the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/glycogen synthase kinase-3β (GSK3β) pathway, has also been recently implicated in the pathology of psychological disorders including chronic stress, depression, and schizophrenia. The Akt pathway also plays a prominent role in the effects of lithium, SSRIs, and other classes of psychotropic drugs (Zhang et al., 2003; Li et al, 2004; Basta-Kaim et al., 2005; Li et al., 2007; Amar et al., 2008; Reddy et al,
2008; Szymanska et al., 2009). For example, increased levels of activated (phosphorylated) Akt (pAkt) have been found in animals subjected to chronic stress (Lee et al., 2006), and the activity of PI3K and Akt were significantly decreased in the occipital cortex of suicide victims when compared to controls (Hsiung et al., 2003). Furthermore, SSRIs increase levels of inactivated (i.e., phosphorylated) GSK3β in the adult mouse frontal cortex, possibly via 5-HT$_{1A}$ receptor activation (Li et al., 2004).

To date, no studies have elucidated the specific pathways or mechanisms of 5-HT$_{1A}$ receptor signaling in the immature hypothalamus. Therefore, the overall purpose of this dissertation is to identify and characterize the 5-HT$_{1A}$ receptor-mediated signaling pathways that are operative in the hypothalamic PVN of peripubertal rats (see figure 2). The determination of hypothalamic 5-HT$_{1A}$ receptor signaling mechanisms, and any aspects that may be unique to the immature brain, should contribute to a better understanding of differences in therapeutic and/or side effects of SSRIs in adult vs. young patients and may identify novel drug targets to treat mood disorders in children and adolescents.

The HYPOTHESIS for this dissertation is that, in the peripubertal rat hypothalamic PVN: (1) 5-HT$_{1A}$ receptors on oxytocin- or CRH-containing neurons stimulate oxytocin and ACTH plasma hormone responses and activate ERK and Akt via different subsets of Ga proteins; (2) different structural classes of agonists may exhibit biased agonism of 5-HT$_{1A}$ receptor-mediated signaling
responses; and (3) 5-HT$_{1A}$ receptor-mediated activation of protein kinase pathways will be resistant to desensitization by SSRIs (in contrast to neuroendocrine responses). The main objectives of the dissertation studies in the peripubertal rat hypothalamic PVN are to determine: (1) if different structural classes of 5-HT$_{1A}$ receptor agonists (i.e., an aminotetralin and/or an azapirone) that stimulate oxytocin and ACTH responses can concomitantly activate the ERK and/or Akt protein kinase pathways; (2) the onset and longevity of the respective 5-HT$_{1A}$ receptor-mediated responses activated by two different classes of agonists; (3) the efficacy and potency of two agonist classes on each of the respective 5-HT$_{1A}$ receptor-mediated responses; (4) potential “agonist-dependent selectivity” or targeting of any specific response pathway by either class of agonist; (5) levels of the respective G$_{a}$ protein subtypes at peripubertal versus younger (prepubertal) and adult ages, (6) the role of PTX-sensitive and PTX-insensitive G$_{a}$ proteins in the respective 5-HT$_{1A}$ receptor-mediated responses; (7) the regulation of hypothalamic G$_{a}$ proteins and 5-HT$_{1A}$ receptor-mediated activation of ERK, Akt, and its downstream effector GSK3β in PVN upon chronic SSRI treatment paradigms (7 or 14 days); and (8) if 5-HT$_{1A}$ receptors can activate ERK in oxytocin- and CRH-containing cells and if the profile of ERK activation is qualitatively similar to that in adult PVN.
Figure 1. 5-HT₁A receptor signaling (A) and SSRI-induced desensitization (B) in the adult rat PVN. In the ADULT PVN, 5-HT₁A receptor activation results in increases in plasma levels of oxytocin and ACTH and increases in pERK levels in PVN. These pathways are mediated by different subsets of Gα proteins and are independent with respect to acute 5-HT₁A receptor activation, yet intact ERK signaling is required for SSRIs to desensitize 5-HT₁A receptor-mediated neuroendocrine responses and decrease hypothalamic levels of Gαz proteins (white dashed line).
Figure 2. Proposed 5-HT1A receptor signaling mechanisms in the PERIPUBERTAL rat PVN. 5-HT1A receptors can potentially activate oxytocin and ACTH plasma hormone responses and protein kinase (ERK and Akt) activation in peripubertal PVN. In these dissertation studies, the potential agonist specificity to activate these respective responses, the role of Gα proteins, cellular localization of ERK responses, and regulation of kinase activity by fluoxetine will be determined.
CHAPTER 2
REVIEW OF RELATED LITERATURE

The serotonergic system

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine neurotransmitter that can also function as a “developmental signal” in early postnatal development (Lauder 1990; Rubenstein 1998). In the brain, serotonergic cell bodies are localized along the midline of the brainstem (medulla, pons, and midbrain) and are organized in 9 distinct nuclei designated B1-B9 by Dahlstrom and Fuxe in 1964. These cell groups generally correspond to the raphe nuclei defined by Taber et al. in 1960, although not all raphe neurons are serotonergic. In the rat, expression of the serotonergic phenotype begins on embryonic day 12 and continues on until embryonic day 15, when generation of 5-HT neurons in the raphe is complete (Lauder 1990; Rubenstein 1998). Serotonergic cells send ascending projections to almost every area of the brain as well as descending projections to the spinal cord. Two prominent and morphologically different ascending pathways arise from the dorsal and median raphe nuclei to innervate the forebrain. Although these pathways have distinct patterns of innervation, projections from both dorsal and median raphe nuclei innervate the hypothalamus (Tork 1990; Hensler 2006). 5-HT mediates its effects via 7
families of receptors (5-HT1-7) containing at least 15 subtypes to regulate a wide variety of functions including behavior, mood, appetite, perception, circadian rhythms, body temperature, and cognitive functions such as memory and learning (Berger et al., 2009).

**5-HT synthesis, release, and reuptake**

5-HT does not pass the blood brain barrier easily; therefore, most brain 5-HT is synthesized within the raphe nuclei. The amino acid precursor to 5-HT, tryptophan, is transported into the central nervous system via a neutral amino acid transporter located on brain capillary endothelial cells (Boado et al., 1999). Brain levels of tryptophan depend on its total concentration in blood, as well as its levels relative to other neutral amino acids. Tryptophan is converted to 5-hydroxytryptophan by tryptophan hydroxylase (TPH). TPH is the rate-limiting enzyme in 5-HT synthesis and is synthesized only in 5-HT-containing cells. TPH exists in two isoforms: TPH1 and TPH2. TPH2 is primarily expressed in brainstem while TPH1 is expressed in the gut, pineal gland, spleen, and thymus (Walther and Bader, 2003). 5-hydroxytryptophan is converted to 5-hydroxytryptamine (5-HT) by the enzyme aromatic amino acid decarboxylase (AAADC). AAADC is not specific to 5-HT-containing cells, as this enzyme can also convert L-dopa to dopamine. 5-HT is packaged into vesicles by the vesicular monoamine transporter 2 (VMAT2) (Hensler in Siegel text, 2006). After synaptic release of 5-HT via exocytosis, the major means of terminating the
signal is reuptake back into the cell by the serotonin transporter (SERT). SERT is localized to neurons, although some astrocytes in culture can take up 5-HT. 5-HT is metabolized to 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase (MAO), which is primarily localized in mitochondria. 5-HT can also be converted to melatonin in the pineal gland (Hensler in Siegel text, 2006).

With regards to developmental levels of some of these regulators of 5-HT synthesis and reuptake, TPH and SERT immunoreactivity in rat raphe neurons are high in early postnatal development but decrease to stable levels by postnatal day 21 (Liu and Wong-Riley, 2010).

**Mechanism of action of selective serotonin reuptake inhibitors (SSRIs)**

Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine (Prozac ®) comparable in effectiveness to other antidepressants, such as tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs), in treating several mood disorders but exhibit a more favorable side effect profile than the TCAs and MAOIs (Hirschfeld 1999; Papakostas and Fava, 2006). SSRIs initially function to block the reuptake of 5-HT by SERT at the synapse, which increases synaptic levels of 5-HT. Therefore, acute administration of SSRIs increases 5-HT and triggers 5-HT₁A autoreceptors (located on the soma and dendrites of 5-HT cells) to inhibit further cell firing (Newman et al., 2004). Upon continued treatment with an SSRI, increased levels of 5-HT desensitizes (i.e., an attenuation of a receptor-mediated response despite the presence of agonist) the inhibitory
somatodendritic 5-HT$_{1A}$ autoreceptors, thereby reestablishing or strengthening 5-HT cell firing. The time necessary for desensitization of 5-HT$_{1A}$ autoreceptors is consistent with the 2-3 week delay in therapeutic response to SSRIs. Desensitization of post-synaptic 5-HT$_{1A}$ receptors on non-serotonergic neurons in other brain regions is also observed with antidepressant drug treatment and may be involved in the long-term therapeutic effect of anxiolytic and antidepressant drugs (Borsini 1994). SSRIs can also attenuate increases in SERT protein levels in the raphe nuclei of chronically stressed rats (Abumaria et al., 2007) and in a rat model of depression (Saitoh et al., 2007).

**The hypothalamic-pituitary-adrenal (HPA) axis**

The serotonergic system exerts significant control over the hypothalamic-pituitary-adrenal (HPA) axis, the main interface between the brain and body in response to both psychological and physiological stressors (Carrasco and Van de Kar, 2003; Sullivan-Hanley and Van de Kar, 2003; Jorgensen 2007; Pompili et al., 2010). Alterations in HPA function have been associated with mood disorders (Modell and Holsboer, 2005; Papiol et al., 2007; Thomson and Craighead, 2008; Bao et al., 2008; Holsboer and Ising, 2010), and antidepressants used to treat these disorders produce changes in HPA function in both humans and animal models (Conti et al., 2007, Lerer et al., 1999, Li et al., 1997, Li et al., 1996, Serres et al., 2000b, Young et al., 2004). The hypothalamus, a major component of the HPA axis, is a limbic structure involved in a number of vital functions such as
circadian rhythms, body temperature regulation, appetite, and the regulation of mood (Adamec 1990, Kruk 1991). The paraventricular nucleus (PVN) is one of several nuclei in the hypothalamus and is a center of control over many neuropeptides/hormones. Oxytocin and corticotrophin releasing hormone (CRH) are two hormones that are subject to serotonergic regulation via 5-HT$_{1A}$ receptors in hypothalamic PVN (Osei-Owusu et al., 2005). 5-HT$_{1A}$ receptors in the PVN mediate hormone release via direct (e.g., oxytocin) and sequential (e.g., CRH $\rightarrow$ adrenocorticotropic hormone (ACTH)) pathways (Armstrong 2004). Measuring plasma levels of ACTH and oxytocin after in vivo injection of 5-HT$_{1A}$ receptor agonists (i.e., “neuroendocrine challenge test”) is a viable means of assessing 5-HT$_{1A}$ receptor function in animal models. Furthermore, neuroendocrine challenge tests are clinically relevant because they may be used in human patients to assess alterations in receptor function due to neuropsychiatric conditions or the effects of psychotropic drugs (Cowen 1998; Lee et al., 2003; Almeida et al., 2010).

The reactivity of the HPA axis is modulated by pubertal development. Basal levels of HPA function remain unchanged, but stress-induced increases in the activation of the HPA axis are significantly elevated and prolonged in peripubertal rats compared to adults. With regards to continued exposure to stressors, peripubertal animals habituate, or adapt, more rapidly than adult animals exposed to the same chronic stressors (Romeo et al., 2006). Lastly,
exposure to stressors during critical periods of maturation can induce long term changes in HPA reactivity (Romeo, 2010). Changes in gonadal hormone levels are responsible for some of these differences between peripubertal and adult animals. For example, testosterone attenuates HPA reactivity in response to an acute stressor (Goel et al., 2011). Testosterone also decreases activation of c-fos in brain regions with inhibitory feedback on the PVN (Williamson et al., 2010; Goel et al., 2011). Therefore, lower levels of testosterone in a peripubertal animal could lead to hyperactivity of the HPA axis.

Neuropeptides and mental illness

**Oxytocin.** Various studies have indicated that neuropeptides may play a major role in the pathology associated with mood disorders and stress responses. Neuropeptides may serve as local, autocrine, paracrine, or regulatory agents in nearby cells (Sawchenko et al., 1984b). Oxytocin, a nine-amino acid residue neuropeptide, was first isolated in 1953 (Du Vigneaud et al., 1953). Oxytocin is mainly synthesized within the soma of magnocellular neurons located in the PVN and supraoptic nucleus in the hypothalamus. Oxytocin is then transported down the axon, packaged in neurosecretory vesicles, and released directly from nerve terminals located in the posterior pituitary, arcuate nucleus, lateral septum, medial amygdaloid nucleus and median eminence. Although oxytocin is most often associated with the effects it has on maternal behavior, pair bonding, and lactation (Marazziti and Catena Dell’Osso 2008), oxytocin is also a
potent neuromodulator and has been found to be anxiolytic in males and females (Newman, 2008; Scantamburlo et al., 2009; Slattery and Neumann 2010). For example, intracerebroventricular infusion of oxytocin into the mouse brain decreases anxiety behavior (Yoshida et al., 2009), and depressed patients show lower plasma oxytocin than controls (Frasch et al., 1995; Ozsoy et al., 2009). Intranasal administration of oxytocin has shown to be clinically useful in certain depressed individuals (Heinrichs and Gaab 2007). Although the anxiolytic effects of oxytocin may be at least partially mediated through the serotonergic system (Yoshida et al., 2009), chronic treatment with SSRIs has no effect on basal plasma levels of oxytocin in rats (Uvnas-Moberg et al., 1999) or in depressed human patients (Ozsoy et al., 2009). However, basal plasma levels of oxytocin may not reflect changes in central levels of oxytocin (Armstrong et al., 1980) that may occur following chronic SSRI treatment.

**CRH.** The interactions of CRH, the HPA axis, and depressive disorders have been more extensively investigated than the involvement of oxytocin in depressive disorders. One of the key players in the HPA axis is CRH, a 41 amino acid residue neuropeptide that was first characterized in 1981 (Vale et al., 1981). Although CRH may access the general circulation (Sawchenko et al., 1984b), the classical mechanism of HPA function is triggered by CRH release from the hypothalamus into the hypothalamo-hypophyseal portal system, where it interacts with one of two types of CRH receptors on corticotrophic cells (CRH-
R1) in the anterior pituitary (Papadimitriou and Priftis, 2009). Upon CRH receptor stimulation, corticotrophic cells release ACTH into the periphery, where it acts on its receptors in the adrenal cortex to synthesize and release corticosteroids (namely cortisol in humans and corticosterone in rats). The corticosteroids act throughout the body to affect a variety of mechanisms necessary to cope with stressors, including regulating plasma glucose levels, metabolism, and CNS effects. There are corticosteroid receptors at multiple levels (e.g., hypothalamus and pituitary) that bind corticosteroids providing a negative feedback mechanism in order to attenuate the stress responses (Barrett 2003).

Although most CRH immunoreactivity is localized to parvocellular neurons in the PVN, CRH is detected in a multitude of other brain regions, which strongly suggests that CRH may act as a neuromodulator in extra-hypothalamic circuits (Swanson et al., 1983; Liposits et al., 1985; Sakanaka et al., 1987). Studies in both humans and animal models have implicated the CRH system in mood disorders and the effects of antidepressants (Nemeroff 1996; Holsboer 1999, 2000; Arborelius et al., 1999; Bale and Vale, 2004; Madaan and Wilson, 2009). Acute fluoxetine elevates CRH in hypophyseal portal plasma and subsequently increases ACTH in plasma in rats (Gibbs and Vale 1983). Chronic stress or intracerebroventricular injection of CRH induces depressive behaviors in a rodent model (Swiergiel et al., 2008). In the human brain, the ratio of the two
subtypes of CRH receptors, CRHR1 to CRHR2, was found to be decreased in the brains of suicide victims as compared to controls (Hiroi et al., 2001). In one human study (albeit one with a limited sample size), a certain genetic variant of the CRHR1 gene was associated with an increased risk for developing a seasonal pattern of depression and an earlier age of onset for the first depressive episode. A genetic variant of the CRHR2 gene predicted a poorer outcome when individuals expressing this variant were given the SSRI citalopram (Papiol et al., 2007). Although these studies represent just a few of the adaptations observed in the CRH system associated with stress and mental illness, it is important to note that most human studies are performed in adults. However, studies of HPA function in children and adolescents are steadily increasing, and some recent studies have also demonstrated HPA alterations in response to stress and mental illness in these younger age groups. For example, children that are bullied (Vaillancourt et al., 2007), have been subjected to sexual abuse (Trickett et al., 2010), have had lost a parent in the September 11, 2001 terrorist attacks (Pfeffer et al., 2007), or who have been displaced by Hurricane Katrina (Vigil et al., 2010) have decreased cortisol levels and risk for depressive disorders. Pituitary gland volume is increased in adolescents with mood disorders as compared to controls (MacMaster et al., 2008). Although these studies provide strong evidence for the involvement of the HPA in depressive behavior in children and adolescents,
preclinical studies of HPA function and the effects of antidepressants in the immature brain are lacking.

**Neuroanatomy of the PVN**

The PVN is localized in the medial hypothalamus and is located rostrally at the level of the optic chiasm. The nucleus is localized laterally to the third ventricle near its dorsal surface (Kiss 1988; Paxinos and Watson, 1998). Each half of the PVN receives input from the contralateral side of the PVN (Silverman et al., 1981). The PVN is a center for complex autonomic and neuroendocrine control of the periphery and central nervous system (Swanson and Sawchenko 1980; Sawchenko and Swanson 1983) and consists of several independently controlled subdivisions—medial and lateral magnocellular (large cell) groups; anterior and medial parvocellular (small cell) cell groups; and ventral, posterior, and dorsomedial cap divisions. The PVN is often subdivided based on the cellular content of neuroactive peptides and neurotransmitters; however, neurons in the PVN are also organized into subnuclei based on common output, size, density, and dendritic morphology (Armstrong 2004).

**Magnocellular neurosecretory cells.** The medial magnocellular region of the PVN contains primarily oxytocin-containing and arginine vasopressin (AVP)-containing cells. AVP-containing cells are localized in a ball-shaped cluster in the lateral magnocellular group located posterior and dorsolateral in the PVN. Oxytocin-containing cells are localized in the medial magnocellular portion
located anteromedially in the PVN, and also form a ring of cells around the ball-shaped AVP neuron group. Magnocellular cells are relatively large (i.e., 20-35 μm) and send their axons to the neural lobe of the hypophysis, forming the hypothalamoneurohypophyseal system. Oxytocin and AVP are transported down these axons for release into the plasma (Armstrong, 2004). Although most CRH-containing cells are localized in the parvocellular cell groups, oxytocin and CRH do moderately colocalize in a discrete anterior part of the magnocellular division of the nucleus (Sawchenko et al., 1984b).

**Parvocellular neurosecretory cells.** Parvocellular groups are chemically diverse (e.g., containing CRH, oxytocin, AVP, and many other neuropeptides) and can be divided into anterior and medial cell groups (Armstrong, 2004). There is also significant colocalization of CRH with AVP in the PVN (Sawchenko et al., 1984a). Cells from each of the anterior and medial subnuclei project to and synapse on the median eminence. Factors released from parvocellular groups are released into the tuberohypophyseal system, where they influence the secretion of anterior lobe hormones (e.g. ACTH).

Although CRH is the classical releasing hormone for ACTH, AVP also plays a role in ACTH stimulation (Papadimitriou and Priftis, 2009). However, although 5-HT regulates the release of AVP, it is most likely stimulated by 5-HT$_{2C}$, 5-HT$_4$, and 5-HT$_7$ receptors (Jorgensen, 2007) and not 5-HT$_{1A}$ receptors (Bagdy et al., 1992; Li et al., 1993).
**Nonendocrine projection cells.** In the PVN, there are also some cell groups that send significant descending projections to the brainstem and spinal cord. These cells are organized into ventral, posterior, and dorsomedial cap subnuclei (Armstrong, 2004). Projections from these cells also innervate some circumventricular organs, pineal gland, and specific nuclei of the thalamus, amygdala, and other areas of the hypothalamus (Pittmann et al., 1981). Retrograde tracing of neurons reveals that the dorsomedial part of the PVN projects to the spinal cord and the dorsolateral cap of the PVN projects to the pituitary, indicating that separate cell populations in the PVN project to pituitary vs. spinal cord (Swanson and Kuypers 1980). Oxytocin receptors have been found on 5-HT containing cells in the raphe nuclei (Yoshida et al., 2009), indicating that oxytocin has the capacity to regulate serotonin release and possibly anxiolytic effects via direct activation of oxytocin receptors in the raphe nuclei.

**Inputs to PVN.** Neurons in the PVN are highly responsive to both peripheral and central stimuli (Saphier and Feldman 1985; Hatton 1990), receiving input from the brain stem, most other hypothalamic areas, the amygdala, and other forebrain areas (Armstrong 2004). The PVN receives significant amounts of monoamine innervation, primarily by 5-HT (raphe nuclei) and norepinephrine (locus coeruleus). Serotonergic projections arise from the raphe nuclei, and fibers from these neurons are found in both oxytocinergic cell groups (Sawchenko et
Considering that 5-HT-containing axons overlap with CRH-immunoreactive neurons in the PVN, it is likely that there are direct synapses between serotonergic terminals and dendrites and cell bodies of CRH cells (Liposits et al., 1987). Dendrites of both parvocellular and magnocellular secretory neurons rarely leave the PVN, but often cross into adjacent subnuclei, where they can locally secrete peptide (Armstrong, 2004).

5-HT$_{1A}$ receptor signaling in the hypothalamic paraventricular nucleus (PVN)

The serotonin 1A (5-HT$_{1A}$) receptor was one of the first 5-HT receptor subtypes to be identified and investigated with respect to its pharmacology, function, and regional distribution in brain. It was also the first 5-HT receptor to be cloned (Albert et al., 1990). 5-HT$_{1A}$ receptors are 7-transmembrane-spanning and are part of the G-protein coupled receptor (GPCR) family. 5-HT$_{1A}$ receptors on serotonergic neurons are localized to the soma and dendrites where they function as inhibitory autoreceptors to regulate cell firing. This population of receptors has generated a great deal of attention since the desensitization of these receptors plays a prominent role in the therapeutic efficacy of various drugs used to treat mood disorders. 5-HT$_{1A}$ receptors are also associated with anxiolytic properties, and knock-out mice lacking this receptor show increased anxiety behavior (Celada et al., 2004; Gross et al., 2002). 5-HT$_{1A}$ receptors also are expressed on non-serotonergic cells in various brain regions such as the hippocampus, frontal cortex, and hypothalamus, where they are postsynaptic...
receptors that can initiate a variety of signal transduction cascades in response to 5-HT or an agonist (Kia et al., 1996a; Kia et al., 1996b; Blier and Ward 2003; Marvin et al., 2010). In the paraventricular nucleus, 5-HT$_{1A}$ receptor immunoreactivity was localized in the medial parvocellular subnucleus as well as in the medial magnocellular subnucleus (Marvin et al., 2010).

5-HT$_{1A}$ receptors have the capacity to couple to the broadest array of G proteins and second messengers. 5-HT$_{1A}$ receptors in hypothalamus can couple to $G_{\alpha i/o}$ and $G_{\alpha z}$ proteins (Garnovskaya et al., 1996; Raymond et al., 1993; Serres et al., 2000a) and localize to at least two populations of cells in the PVN of the hypothalamus: oxytocin- and CRH-containing cells (Zhang et al., 2004). In adult rats, post-synaptic 5-HT$_{1A}$ receptors in the PVN mediate separate and distinct signal transduction pathways mediated by separate subsets of $G_{\alpha}$ proteins (see Chapter 1, figure 1). $G_{\alpha z}$ proteins mediate the 5-HT$_{1A}$ receptor-induced increase of ACTH and oxytocin into the plasma (Li et al., 1994; Li et al., 1993; Serres et al., 2000a; Vicentic et al., 1998; Zhang et al., 2004) while pertussis toxin-sensitive $G_{\alpha i/o}$ proteins mediate the activation of the mitogen activated protein kinase (MAPK) cascade via the phosphorylation of extracellular signal-regulated kinase (ERK) (Chen et al., 2002; Crane et al., 2007; Garcia et al., 2006; Sullivan et al., 2005).
Description of different structural classes of 5-HT$_{1A}$ receptor agonists

There are several structural classes of 5-HT$_{1A}$ receptor agonists. In the current studies, representative members of the aminotetralin class ((+)-8-OH-DPAT) and the azapirone class (tandospirone) are used to determine how these different structural classes compare with regards to potency and efficacy with respect to the different 5-HT$_{1A}$ receptor responses in the peripubertal PVN. More details regarding the structure, molecular weight, etc. is shown in table 1.

(+)-8-OH-DPAT has a high affinity and selectivity for 5-HT$_{1A}$ receptors. Tandospirone also has a high affinity for 5-HT$_{1A}$ receptors, and has similar potency to other members of the azapirone class on the inhibition of neuronal cell firing. Tandospirone is most selective for 5-HT$_{1A}$ than other subtypes of receptors (5-HT$_{1A}$ > 5-HT$_{2C}$ > 5-HT$_{1C}$ > α1 and α2 adrenergic > dopamine D1 and D2 receptors (Hamik et al., 1990). Tandospirone has lower affinity for dopamine D$_2$ receptors than buspirone (Seymour et al., 1990).

In rat septal nuclei cultures, 8-OH-DPAT had full agonist activity on neuron hyperpolarization while buspirone (an azapirone) only had partial activity (Vanden Hooff and Gavan, 1992). 8-OH-DPAT is also more potent than azapirones on the inhibition of cAMP formation in primary culture of mouse hippocampus (Dumuis et al., 1988). In rat and guinea pig hippocampal membranes, buspirone also produced inhibition of forskolin-induced adenylyl cyclase activity, but the maximal inhibition was less than that induced by 5-HT, indicating that
azapirones display partial agonist activity in this response (DeVivo and Maayani, 1986). Tandospirone also displays 60% of the maximal effect of 8-OH-DPAT on inhibition of adenylyl cyclase activity (Hamik et al., 1990). However, tandospirone has been shown to act as a full agonist in inducing 5-HT$_{1A}$ receptor-mediated hypothermia in rats (Millan et al., 1993). These studies demonstrate that the agonist efficacy at 5-HT$_{1A}$ receptors is not dictated exclusively by structural class and may vary with respect to signal transduction pathway associated with 5-HT$_{1A}$ receptors.

**SSRI-induced desensitization of 5-HT$_{1A}$ receptors in the hypothalamic PVN**

Although several studies have linked the clinical efficacy of antidepressants with the time necessary to desensitize 5-HT$_{1A}$ autoreceptors in serotonergic neurons of the hindbrain, time-dependent desensitization of post-synaptic 5-HT$_{1A}$ receptors on non-serotonergic neurons is also observed with long-term treatment and may be involved in the therapeutic effects of anxiolytic and antidepressant drugs (Borsini 1994). Desensitization of 5-HT$_{1A}$ receptors can occur after prolonged elevations of extracellular 5-HT produced by uptake blocking drugs or following prolonged exposure to specific 5-HT$_{1A}$ receptor agonists (Albert et al., 1996). Receptor desensitization may occur after receptor phosphorylation by protein kinase C in a complex with calmodulin (Raymond 1991; Turner et al., 2004). Receptor desensitization differs from drug tolerance in that drug tolerance refers to the attenuation of clinical responsiveness to a drug
after repeated administration, while receptor desensitization refers to the attenuation in receptor signaling after repeated agonist administration (Bourne and von Zastrow, 2004).

*In vivo* studies in adult rats have revealed that chronic SSRI treatment has specific effects on 5-HT$_{1A}$ receptor signaling pathways in the PVN. Chronic exposure to fluoxetine decreases levels of G$_{\alpha z}$ proteins in the PVN and desensitizes 5-HT$_{1A}$ receptor-mediated hormone responses (Raap et al., 1999). However, the G$_{\alpha i/o}$-mediated ERK signaling pathway is not desensitized by long-term SSRI treatment (Jia et al., 2006). In fact, maintaining MAPK signaling is essential for desensitization of 5-HT$_{1A}$ receptor-mediated neuroendocrine responses since blockade of MAPK kinase (MEK), the activating kinase for ERK, abolishes SSRI-mediated desensitization of hormone response and associated reductions in G$_{\alpha z}$ proteins (Jia et al., 2007).

**5-HT receptor regulation in the postnatal period**

5-HT receptor expression occurs very early in fetal development; mRNA transcripts for the 5-HT$_1$ receptor family can be detected as early as embryonic day 14.5 in the mouse brain (Bonnin et al., 2006). Regional and subtype-specific changes in 5-HT receptor mRNA levels continue past the day of birth and persist into the early postnatal period (Basura and Walker, 2000; Volgin et al., 2003). 5-HT$_1$ and 5-HT$_2$ receptor density in the primate brain increases rapidly in the postnatal period and reaches its peak around 2-4 months of age. In general, the
density of cortical 5-HT receptors then begins to decline, reaching adult levels right around the time of puberty (~3 years of age in the primate) (Lidow and Rakic 1992). However, there have been few studies examining developmental changes in 5-HT receptor density in the hypothalamus. There was no change in the density of 5-HT$_{2A}$ receptors in the neonatal rat hypothalamus over the second postnatal week of life, but the density of 5-HT$_{1A}$ receptors increased during this time in the hypothalamus (Ferrari et al., 1999). However, the density or regulation of 5-HT$_{1A}$ receptors in the hypothalamus around the time of puberty has not been determined.

**G proteins**

5-HT$_{1A}$ receptors are part of the G protein coupled receptor family. G proteins are heterotrimeric and mediate signaling from the level of the receptor to a variety of intracellular signaling pathways in the cell. 5-HT$_{1A}$ receptors specifically couple to members of the G$_{ai/o}$ protein family: G$_{ai1}$, G$_{ai2}$, G$_{ai3}$, G$_o$, and G$_z$ (Mannoury la Cour et al., 2006; Raymond et al., 1992; Serres et al., 2000a) and mediate 5-HT$_{1A}$ receptor signaling pathways in the hypothalamic PVN (i.e., oxytocin and ACTH responses and phosphorylation of ERK). Dysfunction of G protein-mediated signaling has been recently linked to psychiatric disorders. The activity and/or protein content of the intermediates of G protein-mediated signaling pathways have shown to be disturbed in patients with bipolar disorder (Friedman and Wang 1996; Spleiss et al., 1998; Dowlatshahi
et al., 1999; Emamghoreishi et al., 2000), schizophrenia (Jope et al., 1998; Yang et al., 1998) and alcohol dependence (Jope et al., 1998). Measuring G protein content in mononuclear leukocytes in patients with mood disorders may be a viable option to measure the effectiveness of various drug treatments (Avissar et al. 2001).

Levels of Ga proteins are known to change throughout several brain regions during early postnatal development. For example, Ga11, Ga12, and Gao proteins steadily increase between postnatal days 1 and 25 in the cortex, thalamus, and hippocampus of the rat brain, whereas Ga3 proteins decrease in these regions over the same time period (Ihnatovych et al., 2002). However, no studies have determined the developmental regulation of these subtypes of Ga proteins or Gaz proteins in the hypothalamus, and no studies have examined the time right around puberty, which is an important period for brain growth and maturation. Any age-dependent differences in the levels of Ga proteins or mechanisms of intracellular signal transduction of these proteins have implications for the function of G protein-mediated signaling in both health and disease states in a population at different stages in development.

The MAPK ERK

Mitogen activated protein kinases (MAPK) make up a family of protein kinases that respond to extracellular stimuli (typically by growth factors). This protein family regulates various cellular activities, including gene expression,
mitosis, differentiation, cell survival/apoptosis, learning, memory, neuroplasticity, and receptor desensitization (Marshall 1995; Pouysségur and Lenormand 2003; Trincavelli et al., 2002). The activation mechanism of extracellular signal-regulated kinase (ERK) is classically initiated by receptor tyrosine kinases, although it can be activated by GPCRs, including 5-HT$_{1A}$ receptors in vitro (Garnovskaya et al., 1996) and in vivo (Chen et al., 2002; Crane et al., 2007; Sullivan et al., 2005). More recently, MAPK function has been implicated in stress and the effects of antidepressants. Acute blockade of MAPK signaling produces a depressive-like phenotype and blocks behavioral actions of antidepressants in mice (Duman et al., 2007; Qi et al., 2009), while chronic corticosterone treatment of mice increases depressive-like behaviors and reduces levels of activated ERK in the dentate gyrus (Gourley et al., 2008). Also, acute stress alters levels of activated ERK in several regions of the rat brain (Shen et al., 2004; Qi et al., 2006), which can be reversed by fluoxetine (Qi et al., 2008). These findings are consistent with findings from our laboratory demonstrating that, in adult rat hypothalamus, ERK activation is required for SSRIs to desensitize 5-HT$_{1A}$ receptor signaling of neuroendocrine responses (Jia et al., 2007). Furthermore, a small post-mortem study determined that several intermediate proteins of ERK signaling as well as ERK itself, were decreased in content and/or activity in the frontal cortex and hippocampus in patients with psychiatric disorders, including schizophrenia and major depressive disorder (Yuan et al.,
2010) and in subjects who had committed suicide (Dwivedi et al., 2001; Dwivedi et al., 2006). Similarly, the activity of MEK (the upstream activating kinase for ERK), but not relative protein levels, was decreased in the frontal cortex and hippocampus of suicide victims when compared to controls (Dwivedi et al., 2009).

**The PI3K/Akt/GSK3 signaling pathway**

Multiple lines of evidence indicate that the PI3K/Akt/GSK3 pathway plays a role in the pathology of psychological disorders including chronic stress, depression, and schizophrenia (Beaulieu et al., 2009). Akt (protein kinase B, previously known as RAC-PK (related to PKA and C protein kinases)) is a protein kinase involved with cell survival, apoptosis, protein synthesis, insulin signaling, and glucose transport (Franke et al., 2003). Akt, a 57 kDa serine/threonine protein kinases, was first cloned in 1991 (Coffer and Woodgett, 1991; Jones et al., 1991; Bellacosa et al., 1991). Akt exists in three isoforms, Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ), with Akt1 having the highest expression in brain tissue (Coffer and Woodgett, 1991). Akt can be activated by receptor tyrosine kinases or by G protein-coupled receptors (GPCRs). In either case, receptor activation activates phosphoinositide 3-kinase (PI3K), which catalyzes the formation of the phosphatidylinositol(3,4,5)P3 (PIP3) molecule. PIP3 attracts phosphoinositide dependent kinase 1 (PDK1) and Akt to the cell membrane, where PDK1 phosphorylates Akt at Thr308 (Alessi et al., 1997; Stephens et al.,
Akt also must be phosphorylated at Ser473 for full activation of the protein (Alessi et al., 1996). The kinase mammalian target of rapamycin (mTOR) is considered to be the candidate responsible for this phosphorylation (Sarbassov et al., 2005), although other kinases like integrin-linked kinase may function to phosphorylate Akt at Ser473 in transfected cell lines (Delcommenne et al., 1998). Although phosphorylation of Akt at Thr308 and Ser473 appear to be autonomously regulated (Coffer et al., 1998), phosphorylation at Ser473 may assist in the recruitment of Akt to the plasma membrane and to subsequent phosphorylation at Thr308 by PDK1 (Andjelkovic et al., 1997; Scheid and Woodgett, 2003). Downstream, activated Akt phosphorylates and inhibits the constitutively active glycogen synthase kinase 3 (GSK3), an enzyme that regulates the function of a multitude of proteins, including metabolic, signaling, and structural proteins (Grimes and Jope 2001). There are 2 isoforms of GSK3, GSK3α and GSK3β, which are both expressed at high levels in the brain (Woodgett 1990).

The Akt signaling pathway been linked to the pathology of mood disorders and the effects of antidepressants and other psychotropic drugs in both humans and animal models. For example, the activities of PI3K and Akt were significantly decreased in the occipital cortex of suicide victims when compared to controls (Hsiung et al., 2003). Chronic stress increases levels of activated (phosphorylated) Akt (pAkt) in the rat hypothalamus, hippocampus, and
striatum, with the largest increases observed in the hypothalamus (Lee et al., 2006). Alterations were observed in both the activity of Akt and GSK3 in schizophrenic patients (Emamian et al., 2004) and in a mouse model of schizophrenia (Beaulieu et al., 2004). Furthermore, acute injection with SSRIs increases the levels of phosphorylated GSK3, which serves to inactivate this kinase (Li et al., 2004). Lithium, commonly used in the treatment of bipolar disorder, has been shown to activate Akt and/or inhibit the activation of GSK3 in vitro and in vivo (Klein and Melton, 1996; Chalecka-Franaszek and Chuang, 1999; DeSarno et al., 2002; Zhang et al., 2003; O’Brien et al., 2004; Gould et al., 2004). Other psychotropic drugs have also been shown to regulate this pathway, including sodium valproate, used in the treatment of bipolar disorder (De Sarno et al., 2002; Roh et al., 2005); imipramine, a tricyclic antidepressant (Li et al., 2004; Basta-Kaim et al., 2005; Roh et al., 2005); risperidone, an atypical antipsychotic (Li et al., 2007); and sertraline, fluoxetine, and fluvoxamine, all SSRIs (Reddy et al., 2008; Fatemi et al., 2009; Nakano et al., 2010).

5-HT₁A receptor agonists increase the phosphorylation of Akt in both neuronal (Adayev et al., 1999; Cowen et al., 2005) and non-neuronal (Hsiung et al., 2005) cell cultures as well as in adult rat hippocampus in vivo (Polter et al., 2010). 5-HT₁A receptor activity has also been shown to stimulate mitochondrial movement in hippocampal axons in vitro, possibly via activation of Akt and blockade of GSK3β (Chen et al., 2007). Acute injection with the SSRI fluoxetine
has also been shown to increase phosphorylated GSK3β in the frontal cortex \textit{in vivo}, possibly via 5-HT_{1A} receptor activation (Li et al., 2004).

**Rationale for developmental time point chosen for these studies**

In this dissertation, a “peripubertal” time period (around postnatal day 42) was chosen to further examine the 5-HT_{1A} receptor signaling and drug-induced regulation of this receptor in the hypothalamic PVN. This age roughly corresponds to “adolescence” in humans (Spear 2000). Endocrine studies have established that sexual maturation in the male rat begins around postnatal days 25-30 and is usually complete by postnatal days 50-60 (Pak and Handa, 2007). We have also chosen this timeframe due the extensive amount of brain changes, neuroplasticity, and behavioral changes occurring during this time. For example, prior to and during puberty, monoamine receptors and synapses (e.g., serotonin, dopamine) are being overproduced in the brain, which are later pruned to adult levels (Andersen 2003). Axonal projections from monoamine cell bodies also reach adult levels around the time of puberty (Andersen and Navalta, 2004). There are also transformations in the adolescent brain with regards to (1) reductions in energy utilization (Spear, 2000), (2) increases in cortical white matter (Villablanca et al., 2000; Sowell et al., 2003), and (3) region-dependent decreases in the relative size of gray matter (Geidd et al., 1999; Rapoport et al., 1999). These changes contribute to the transformation of “the more energy utilizing, seemingly less efficient brain of the child into a more
rapidly communicative and more energy efficient brain of the adult” (Spear, 2007). Considering these global and regional changes, it may be possible that 5-HT$_{1A}$ receptor signaling may undergo transitional changes during this time frame and differ mechanistically from that observed in the adult brain.

It is important to establish the parameters that constitute “normal” functional processes that exist around the time of sexual maturation because both human and rodent studies have provided evidence that stress before sexual maturation induces permanent changes to the brain and body. For example, rats exposed to early life stress displayed blunted responses of ERK activation along with other markers of synaptic signaling in adulthood (Musazzi et al., 2010) and also displayed disturbances in other MAPK signaling pathways that were attenuated with antidepressant treatment (Budziszewska et al., 2010). Chronic social stress of peripubertal rats alters fat distribution in adulthood, but these changes were reversed with SSRI treatment (Schmidt et al., 2009). In human studies, exposure to early life stress before maturity (death of a close relative, parental depression or divorce, etc.) can increase sensitivity to depression upon later stressful events (Bouma et al., 2008), increase immune activity to psychosocial stress, particularly with those suffering from major depressive disorder (Pace et al., 2006), and have long-lasting effects on the HPA axis such as decreased cortisol responses as adults (Meinlschmidt and Heim 2005). Individuals exposed to early life stress who later developed depressive disorders as adults had increased plasma ACTH
responses to challenge with dexamethasone/CRH (a type of neuroendocrine challenge test), and the increases were associated with the severity, onset, and duration of early abuse (Heim et al., 2008). The long term implications for studying brain changes induced by stress or drug treatment (not to mention the functioning of the brain in a basal state) is that clinical interventions or therapies could be designed to optimally treat individuals with specific pathologies (Agid et al., 2000; Heim et al., 2004).

**Summary and clinical significance**

Depressive disorders can occur in up to 4.6% of children and 8.0% of adolescents (Nobile et al., 2003). SSRIs have been shown to be effective in treating childhood and adolescent clinical disorders such as depression, obsessive-compulsive disorders, eating disorders, Tourette’s syndrome, autism, and panic disorder (Cohen 2007; Rushton and Whitmire, 2001; Wong et al., 2004). Although the effectiveness and side effect profile of SSRI use in children and adolescent populations is still highly debatable, fluoxetine (Prozac®) is still an effective option for treating clinical disorders in younger populations. Therefore, at this point in time, the benefits of these drugs may outweigh the risks, and it is highly likely that SSRIs will continue to be prescribed to children and adolescents. Given the involvement of 5-HT, the HPA axis, and protein kinases in the pathogenesis of mood disorders and the effects of antidepressants in adults, more research is necessary to determine the role these systems may play.
in the occurrence and treatment of mood disorders in younger individuals. Determining the aspects of these systems that differ during development may also provide better or more effective targets for the treatment of mood disorders in children and adolescents. Although 5-HT$_{1A}$ receptor signaling and drug-induced regulation of 5-HT$_{1A}$ receptors have been extensively investigated in adult animals, to date no studies have elucidated the specific pathways or mechanisms of 5-HT$_{1A}$ receptor signaling and drug-induced desensitization in the peripubertal hypothalamic PVN.
<table>
<thead>
<tr>
<th></th>
<th>Aminotetralin</th>
<th>Azapirone</th>
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<tbody>
<tr>
<td>Drug used in these studies</td>
<td>(+)8-OH-DPAT</td>
<td>Tandospirone</td>
</tr>
<tr>
<td>Structure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activity at 5-HT$_{1A}$ receptor-mediated inhibition of adenyl cyclase</td>
<td>Full</td>
<td>Partial</td>
</tr>
<tr>
<td>Molecular weight</td>
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<td>383.49 g/mol</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C$<em>{16}$H$</em>{25}$NO</td>
<td>C$<em>{21}$H$</em>{29}$N$_5$O$_2$</td>
</tr>
<tr>
<td>Usage</td>
<td>Prototypical 5-HT$_{1A}$ receptor agonist (preclinical); not approved for clinical use</td>
<td>One of several preclinically used azapirones; anxiolytic, antipsychotic augmentation (Japan)</td>
</tr>
</tbody>
</table>

Table 1. Comparison of the properties of the 5-HT$_{1A}$ receptor agonists used in the current studies.
CHAPTER 3

SEROTONIN 1A RECEPTORS ACTIVATE MULTIPLE SIGNALING RESPONSES IN THE PERIPUBERTAL RAT HYPOTHALAMIC PARAVENTRICULAR NUCLEUS: EFFECTS OF DIFFERENT STRUCTURAL CLASSES OF AGONISTS ON THE TIME- AND DOSE-DEPENDENT STIMULATION OF NEUROENDOCRINE RESPONSES AND ACTIVATION OF EXTRACELLULAR SIGNAL-REGULATED KINASE.

ABSTRACT

In the adult rat hypothalamic paraventricular nucleus (PVN), (+)8-OH-DPAT (an aminotetralin full agonist) and tandospirone (an azapirone partial agonist) increase plasma levels of oxytocin and ACTH and activate extracellular signal regulated kinase (ERK) in the adult PVN (Sullivan et al., 2005; Crane et al., 2007). The present studies investigated 5-HT1A receptor signaling in the peripubertal rat hypothalamic PVN to determine: (1) if different structural classes of 5-HT1A receptor agonists can mediate signaling of both hormone release and kinase activation; (2) the onset and longevity of the respective 5-HT1A receptor-mediated responses; (3) the efficacy and potency of (+)8-OH-DPAT and tandospirone in activating the respective 5-HT1A receptor-mediated responses, (4) potential agonist-dependent targeting of any specific response, and (5) an optimal agonist dose and post-injection time to investigate the multiple 5-HT1A receptor-mediated responses in subsequent studies. With respect to the time-dependent
activation of 5-HT_{1A} receptor-mediated responses, (+)8-OH-DPAT (0.20 mg/kg) and tandospirone (10 mg/kg) induced rapid (≤ 5 minutes) increases in plasma levels of oxytocin that were comparable (~200 pg/ml) and maintained for 15 minutes before declining to baseline (> 30 minutes). Both 5-HT_{1A} agonists also induced rapid (≤ 5 minutes) increases in plasma levels of ACTH that reached comparable maximal levels (~1000 pg/ml) at 10 minutes and remained elevated throughout the 30 minute testing period. (+)8-OH-DPAT and tandospirone also produced rapid (≤ 5 minutes) increases in activated (i.e., phosphorylated) ERK (pERK) that persisted for at least 30 minutes. The dose response data indicate that both drugs exhibited comparable maximal efficacies in elevating plasma oxytocin, but (+)8-OH-DPAT was a more potent agonist than tandospirone. The maximal efficacies of (+)8-OH-DPAT and tandospirone on ACTH responses were also comparable. With respect to ERK activation in the PVN, (+)8-OH-DPAT was more potent than tandospirone and maximally effective doses of the aminotetralin or azapirone agonists produced comparable increases in pERK. In summary, these studies in peripubertal hypothalamic PVN demonstrated different structural classes of 5-HT_{1A} receptor agonists activated both neuroendocrine responses (i.e., oxytocin and ACTH) from different cell types and concomitantly kinase (i.e., pERK) responses, indicating no agonist-dependent targeting of responses. While (+)8-OH-DPAT was more potent than tandospirone at each of the respective responses, both structural classes of 5-
HT₁A receptor agonists produced rapid and prolonged activation of neuroendocrine and kinase responses and similar maximal efficacies.

**INTRODUCTION**

In adult rats, 5-HT₁A receptors are localized on oxytocin-containing and corticotrophin releasing hormone (CRH)-containing cells (Zhang et al., 2004), and activation of these receptors produces respective increases in plasma levels of oxytocin and ACTH hormones (Bagdy and Kalogeras, 1993; Gilbert et al., 1988; Vicentic et al., 1998). 5-HT₁A receptor stimulation concomitantly activates the mitogen-activated protein kinase (MAPK) cascade in the adult PVN (Crane et al., 2007; Sullivan et al., 2005). The 5-HT₁A receptor-mediated activation of ERK by the aminotetralin full agonist (+)8-OH-DPAT is rapid but transient, since levels of activated ERK are increased at 5 minutes but return to basal levels by 15 minutes post-injection (Crane et al., 2007). In contrast, 8-OH-DPAT-induced elevations in plasma hormone levels of oxytocin and ACTH are more prolonged, since hormone levels reach a peak at 15 minutes and remain significantly elevated 30 minutes after agonist injection (Di Sciuullo et al., 1990; D'Souza et al., 2004). In addition to differences in onset and longevity, the respective 5-HT₁A receptor-mediated responses also differ in terms of the dose-dependence of activation. In the adult, 5-HT₁A receptor-mediated oxytocin and ACTH neuroendocrine responses occur at doses of (+)8-OH-DPAT lower than doses required to significantly increase the levels of pERK in the PVN. Tandospirone,
an azapirone “partial” agonist at postsynaptic 5-HT\textsubscript{1A} receptors, also increases plasma levels of oxytocin and ACTH and increases pERK in the adult rat PVN (Sullivan et al., 2005). However, it remains to be determined if this class of 5-HT\textsubscript{1A} receptor agonist exhibits potency, efficacy, and a time-course of activation comparable to (+)8-OH-DPAT at each of the respective 5-HT\textsubscript{1A} receptor-mediated responses. We have previously reported (Garcia et al., 2006) that hypothalamic 5-HT\textsubscript{1A} receptors in the adult PVN mediate neuroendocrine versus kinase responses via different subsets of G\textsubscript{\alpha} proteins and that these responses can be activated independent of each other. Consequently, different classes of receptor agonists may preferentially or exclusively activate one of these multiple 5-HT\textsubscript{1A} receptor-mediated responses. This is referred to as “biased agonism,” a phenomenon observed in receptor systems where different classes of receptor agonists may induce a variety of conformational changes in a receptor that facilitates signaling via one or more distinct pathways (Kenakin 1995; Berg et al., 1998; Galandrin and Bouvier, 2006; Rajagopal et al., 2010; Whalen et al., 2011). For example, in a cell line expressing 5-HT\textsubscript{2C} receptors, the piperazine derivative 3-trifluoromethylphenyl-piperazine preferentially activates the phospholipase C pathway, whereas lysergic acid diethylamide (LSD), a member of the ergoline family, preferentially activates the phospholipase A\textsubscript{2} pathway (Berg et al., 1998). To date, no \textit{in vivo} studies have investigated the phenomenon of “biased agonism” for 5-HT\textsubscript{1A} receptor-mediated signaling pathways in hypothalamic
PVN at any age. Furthermore, no studies have examined, \textit{in vivo}, the multiplicity of responses mediated by 5-HT$_{1A}$ receptors nor the time-course, efficacy, and potency of different classes of agonists in simulating respective neuroendocrine and kinase responses in the peripubertal hypothalamic PVN.

The first experiment described in this chapter will determine the time-course of classes of 5-HT$_{1A}$ receptor agonists that differ in chemical structure \((+)^8\text{-OH-DPAT}, \text{an aminotetralin, and tandospirone, an azapirone}) to stimulate: (A) increases in plasma oxytocin, (B) increases in plasma ACTH, and (C) increases in pERK in the peripubertal rat hypothalamic PVN. The second experiment will test the dose-dependent effects of the two different agonists on signaling of 5-HT$_{1A}$ receptor-mediated: (A) oxytocin hormone responses, (B) ACTH hormone responses, and (C) increases in pERK in the peripubertal rat hypothalamic PVN.

These studies will provide information regarding: (1) the onset and longevity of the respective 5-HT$_{1A}$ receptor-mediated responses, (2) differences in agonist potency and efficacy for the respective 5-HT$_{1A}$ receptor-mediated responses, (3) potential agonist-dependent preference or exclusive activation of any of the respective 5-HT$_{1A}$ receptor-mediated pathways in the hypothalamic PVN of peripubertal rats, and (4) an optimal agonist dose and post-injection time to obtain multiple 5-HT$_{1A}$ receptor-mediated responses for subsequent studies.
MATERIALS AND EXPERIMENTAL PROCEDURES (for detailed methods, see Chapter 7)

Animals. Peripubertal male Sprague-Dawley rats (postnatal day 35; 100-125 g) were purchased from Harlan (Indianapolis, IN). The determination of post-natal day zero (day of birth) was carried out by the supplier.

Drugs. (+)-8-Hydroxy-2-(di-n-propylamino)tetrалin hydrobromide [(+)-8-OH-DPAT] was purchased from Tocris Cookson Inc. (Ellisville, MO). Vandospirone citrate was a generous gift of Dr. K. Matusubara, Asahikawa Medical College, Japan. All drugs were dissolved in saline (0.9% NaCl). For the dose-response experiment, the highest dose of drug was dissolved in saline and lower concentrations were made by serial dilution. The drugs were injected at a volume of 1 ml/kg and were freshly prepared immediately prior to the injections.

Experiment 1: Time-dependent activation of 5-HT1A receptor-mediated responses. At least 3 days of handling preceded any injections. Peripubertal male Sprague-Dawley rats (post-natal day 42) were injected with either saline (1 ml/kg, s.c.), the aminotetralin full agonist (+)-8-OH-DPAT (0.20 mg/kg, s.c.), or the azapirone partial agonist tandospirone (10 mg/kg, s.c.). These agonist doses have been shown to significantly elevate plasma oxytocin and ACTH concentrations in peripubertal rats (Battaglia et al., 2002; Petrunich et al., 2008) and significantly increase plasma levels of oxytocin and ACTH and increase pERK in the PVN of adult rats (Crane et al., 2007; Sullivan et al., 2005).
animals were sacrificed by decapitation at 5, 10, 15, 20, or 30 minutes post-injection. The brains were carefully removed from the skull within one minute of the decapitation and were quickly frozen by submersion in chilled 2-methylbutane (-35°C) for 1 minute and then subsequently kept in dry ice for an additional 10 minutes. Each brain was then securely wrapped and stored at -80°C prior to dissection for Western blot studies for the determination of pERK/ERK levels in the PVN. The trunk blood was collected and prepared for subsequent determination of plasma levels of oxytocin, ACTH, and testosterone.

**Experiment 2: Dose-dependent activation of 5-HT$_{1A}$ receptor-mediated responses.** At least 3 days of handling preceded any injections. Peripubertal male Sprague-Dawley rats (post-natal day 42) were injected with either saline (1 ml/kg, s.c.), (+)8-OH-DPAT (0.04, 0.10, 0.20, or 0.50 mg/kg, s.c.), or tandospirone (1, 3, 10, or 20 mg/kg, s.c.) and were sacrificed by decapitation at 10 minutes post-injection. These agonist doses have been shown to produce dose-dependent responses in plasma levels of oxytocin and ACTH and pERK in the PVN of adult rats (Battaglia et al., 2009; Sullivan et al., 2005). The brains were carefully removed from the skull within one minute of the decapitation and were quickly frozen by submersion in chilled 2-methylbutane (-35°C) for 1 minute and then subsequently kept in dry ice for an additional 10 minutes. Each brain was then securely wrapped and stored at -80°C prior to dissection for Western blot studies for the determination of pERK/ERK levels in the PVN. The trunk blood was
collected and prepared for subsequent determination of plasma levels of oxytocin, ACTH, and testosterone.

RESULTS

1. **Experiment 1: Time-dependent activation of 5-HT\textsubscript{1A} receptor-mediated responses.** The specific objectives of this experiment were: (1) to determine the onset and longevity of the 5-HT\textsubscript{1A} receptor-mediated increases in (A) plasma levels of oxytocin, (B) plasma levels of ACTH, and (C) phosphorylation of ERK in PVN; (2) to identify potential agonist-dependent differences in onset and longevity of the respective 5-HT\textsubscript{1A} receptor-mediated signaling pathways; and (3) establish one optimal post-injection time (for use in subsequent studies) to measure agonist-induced increases in these functional markers.

A. **Time-course of (+)8-OH-DPAT and tandospirone-induced increases in plasma oxytocin in the peripubertal rat.** As shown in figure 3, (+)8-OH-DPAT (0.20 mg/kg) and tandospirone (10 mg/kg) induced rapid (≤ 5 minutes) and comparable increases in plasma levels of oxytocin that were maintained for 15 minutes before gradually approaching baseline. Although (+)8-OH-DPAT-induced increases in plasma oxytocin attenuate more rapidly than tandospirone, plasma oxytocin responses induced by either agonist did not return to baseline within the time period tested.

The two-way ANOVA for oxytocin indicated a significant main effect of acute challenge injection (saline, (+)8-OH-DPAT, or tandospirone) \[F_{2,115} = 269.13, \ p <\]
0.0001] and a significant main effect of post-injection time \([F_{4,115} = 24.46, p < 0.0001]\). There was also a significant interaction between acute challenge injection and post-injection time \([F_{8,115} = 8.51, p < 0.0001]\). A Newman-Keuls’ test indicated that (+)8-OH-DPAT increased plasma oxytocin levels above saline at 5, 10, 15, 20 minutes (\(p < 0.01\)) and 30 minutes (\(p < 0.05\)). Tandospirone increased plasma oxytocin levels above saline at 5, 10, 15, 20, and 30 minutes (\(p < 0.01\)). These data indicate that (+)8-OH-DPAT (0.20 mg/kg) and tandospirone (10 mg/kg) elicit comparable changes in plasma oxytocin with respect to the magnitude and general overall time-course of the response (although (+)8-OH-DPAT approached baseline more rapidly than tandospirone).

B. **Time-course of (+)8-OH-DPAT and tandospirone-induced increases in plasma ACTH in the peripubertal rat.** As shown in figure 4, (+)8-OH-DPAT (0.20 mg/kg) and tandospirone (10 mg/kg) induced rapid (\(\leq 5\) minutes) increases in plasma levels of ACTH that reached comparable maximal levels at 10 minutes and remained elevated throughout the testing period.

The two-way ANOVA for ACTH indicated a significant main effect of acute challenge injection (saline, (+)8-OH-DPAT, or tandospirone) \([F_{2,120} = 412.46, p < 0.0001]\) and a significant main effect of post-injection time \([F_{4,120} = 10.18, p < 0.0001}\). There was also a significant interaction between acute challenge injection and post-injection time \([F_{8,120} = 3.04, p = 0.004}\). A Newman-Keuls’ test indicated that (+)8-OH-DPAT significantly increased (\(p < 0.01\)) plasma ACTH
levels above saline at 5, 10, 15, 20, and 30 minutes. Tandospirone also significantly increased (p < 0.01) plasma ACTH levels above saline at 5, 10, 15, 20, and 30 minutes. These data indicate that (+)8-OH-DPAT (0.20 mg/kg) and tandospirone (10 mg/kg) elicit comparable changes in plasma levels of ACTH with respect to the magnitude and overall time-course of the response (e.g., peak levels around 10 minutes with a slight attenuation and plateau for subsequent time points for both drugs).

C. **Time-course of (+)8-OH-DPAT-induced increases in phosphorylated ERK in the peripubertal PVN.** As shown in figure 5, (+)8-OH-DPAT (0.20 mg/kg) induced a rapid (≤ 5 minutes) increase in pERK levels in the PVN that was maintained for 20 minutes before declining to baseline (> 30 minutes).

The two-way ANOVA for (+)8-OH-DPAT-induced increases of pERK in the PVN indicated a significant main effect of acute challenge injection (saline or (+)8-OH-DPAT) [F_{1,78} = 43.92, p < 0.0001] but no significant main effect of post-injection time [F_{4,78} = 1.02, p = 0.4022]. There was also no significant interaction between acute challenge injection and post-injection time [F_{4,78} = 1.02, p = 0.4022]. A Newman-Keuls’ test indicated that (+)8-OH-DPAT significantly increased (p < 0.01) pERK in the PVN over saline at 5, 10, 15, and 20 minutes. These data indicate that (+)8-OH-DPAT rapidly increases activated ERK in the peripubertal PVN, and that this response is sustained for a longer response time than previously published for the adult PVN (Crane et al., 2007).
D. Time-course of tandospirone-induced increases in phosphorylated ERK in the peripubertal PVN. As shown in figure 6, tandospirone (10 mg/kg) induced a rapid (≤ 5 minutes) increase in pERK in the PVN that was maintained throughout the testing period, although the magnitude of the response induced by tandospirone (↑ ~50% above basal) was much less than that induced by (+)8-OH-DPAT (↑ ~200% above basal, see figure 5).

The two-way ANOVA for tandospirone-induced increases of pERK in the PVN indicated a significant main effect of acute challenge injection (saline or tandospirone) \[F_{1,75} = 25.87, p < 0.0001\] but no significant main effect of post-injection time \[F_{4,75} = 0.05, p = 0.944\]. There was also no significant interaction between acute challenge injection and post-injection time \[F_{4,75} = 0.05, p = 0.944\]. A Newman-Keuls’ test indicated that tandospirone significantly increased (p < 0.05) pERK over saline at 5, 10, and 20 minutes.

E. Optimal sacrifice time for future experiments. These results indicate that a sacrifice time of 10 minutes post-injection can be used in subsequent studies to measure robust increases in 5-HT$_{1A}$ receptor-mediated increases in plasma oxytocin and ACTH and increases in pERK in the PVN of peripubertal rats produced by injection with (+)8-OH-DPAT.

F. Single injection of (+)8-OH-DPAT or tandospirone does not alter testosterone levels in the peripubertal rat. As shown in figure 7, neither (+)8-OH-DPAT (0.20
mg/kg) nor tandospirone (10 mg/kg) had any effect on plasma levels of testosterone at any time point tested.

The two-way ANOVA for plasma testosterone levels indicated no significant main effect of acute challenge injection (saline, (+)8-OH-DPAT, or tandospirone) [F_{2,97} = 0.42, p = 0.66] and no significant main effect of post-injection time [F_{4,97} = 1.94, p = 0.11]. There was also no significant interaction between acute challenge injection and post-injection time [F_{8,97} = 0.68, p = 0.71]. These data indicate that the stage of sexual maturation was comparable between treatment groups and that the \textit{in vivo} challenge injections and sacrifice times did not alter the testosterone levels in these animals.

2. \textbf{Experiment 2: Dose-dependent activation of 5-HT_{1A} receptor-mediated responses.} The objectives of this experiment were: (1) to determine potency and efficacy of agonists from two different structural drug classes ((+)-8-OH-DPAT, an amiotetralin and tandospirone, an azapirone) on (A) oxytocin hormone responses, (B) ACTH hormone responses, and (C) phosphorylation of ERK in PVN; (2) to determine if either agonist preferentially activates any one of the multiple 5-HT_{1A} receptor-mediated responses; and (3) to establish one optimal dose of (+)-8-OH-DPAT that evokes 5-HT_{1A} receptor-mediated increases in plasma levels of oxytocin and ACTH and increases pERK in the PVN (to be used in subsequent studies).
A. Dose-response of (+)8-OH-DPAT and tandospirone on plasma levels of oxytocin in the peripubertal rat. As shown in figure 8, (+)8-OH-DPAT and tandospirone dose-dependently increase plasma oxytocin levels above those in saline-challenged peripubertal rats. The maximal responses induced by both agonists were similar; however, (+)8-OH-DPAT is more potent agonist than tandospirone, since lower doses of (+)8-OH-DPAT were sufficient to induce oxytocin responses. In addition, the dosing range (less than 1 log unit) required by (+)8-OH-DPAT to achieve a maximal effect on oxytocin responses was much narrower than the dosing range (over 1 log unit) required by tandospirone for maximal effects on plasma levels of oxytocin.

The two-way ANOVA for oxytocin indicated a significant main effect of acute challenge injection (saline, (+)8-OH-DPAT, or tandospirone) \([F_{2,103} = 182.82, p < 0.0001]\) and a significant main effect of agonist dose \([F_{3,103} = 44.24, p < 0.0001]\). There was also a significant interaction between acute challenge injection and agonist dose \([F_{6,103} = 13.73, p < 0.0001]\). A Newman-Keuls’ test indicated that (+)8-OH-DPAT significantly increased \((p < 0.01)\) plasma oxytocin levels above saline-injected controls at 0.10, 0.20, and 0.50 mg/kg, but not 0.04 mg/kg. Tandospirone significantly increased \((p < 0.01)\) plasma oxytocin levels above saline-injected controls at all doses tested (1, 3, 10, and 20 mg/kg). These data suggest that the highest doses of each agonist elicit similar maximal responses with regard to 5-HT_{1A} receptor-mediated increases of plasma oxytocin levels,
although the potency and dosing range necessary to induce maximal responses differ between these two drugs.

B. Dose-response of (+)8-OH-DPAT and tandospirone on plasma levels of ACTH the peripubertal rat. As shown in figure 9, all tested doses of (+)8-OH-DPAT and tandospirone increased plasma ACTH levels over saline-challenged peripubertal rats. The maximal responses induced by both agonists were similar, with (+)8-OH-DPAT inducing a dose-dependent increase in plasma ACTH over a narrow dosing range (less than 1 log unit). As all tested doses of tandospirone elicited a maximal response, neither the potency nor the dosing range could be determined for tandospirone.

The two-way ANOVA for ACTH indicated a significant main effect of acute challenge injection (saline, (+)8-OH-DPAT, or tandospirone) \( [F_{2,102} = 526.44, \ p < 0.0001] \) and a significant main effect of agonist dose \( [F_{3,102} = 43.20, \ p < 0.0001] \). There was also a significant interaction between acute challenge injection and agonist dose \( [F_{6,102} = 23.09, \ p < 0.0001] \). A Newman-Keuls’ test indicated that (+)8-OH-DPAT significantly increased plasma ACTH levels over saline at 0.04 mg/kg (\( p < 0.05 \)), 0.10, 0.20, and 0.50 mg/kg (\( p < 0.01 \)). Tandospirone significantly increased (\( p < 0.01 \)) plasma ACTH levels above saline-injected controls at all doses tested (1, 3, 10, and 20 mg/kg). These data suggest that the highest doses of each agonist elicit similar maximal responses (\( E_{\text{max}} \)) with regards to 5-HT\textsubscript{1A} receptor-mediated increases of plasma ACTH levels, although the
potency and dosing ranges was not obvious for tandospirone and cannot be easily compared to the values obtained for (+)8-OH-DPAT.

C. **Dose-response of (+)8-OH-DPAT on levels of pERK in peripubertal rat hypothalamic PVN.** As shown in figure 10, the highest doses of (+)8-OH-DPAT tested increased pERK levels in the peripubertal PVN over saline-challenge. Within the dosing range tested, these responses approximate the maximal response, although the additional data points at higher doses would better substantiate the respective $E_{\text{max}}$ values. (+)8-OH-DPAT is a potent agonist on 5-HT$_{1A}$ receptor-mediated pERK responses in the PVN, with a more narrow dosing range ($\leq$ 1 log unit).

The two-way ANOVA for (+)8-OH-DPAT-induced increases of pERK in the PVN indicated a significant main effect of acute challenge injection (saline or (+)8-OH-DPAT) [$F_{1,71} = 11.55, p = 0.0012$] and a significant main effect of agonist dose [$F_{3,71} = 4.90, p = 0.0039$]. There was also a significant interaction between acute challenge injection and agonist dose [$F_{3,71} = 4.90, p = 0.0039$]. A Newman-Keuls’ test indicated that (+)8-OH-DPAT increased levels of pERK in the PVN over saline at 0.20 mg/kg ($p < 0.05$) and at 0.50 mg/kg ($p < 0.01$).

D. **Dose-response of (+)8-OH-DPAT and tandospirone on levels of pERK in peripubertal rat hypothalamic PVN.** As shown in figure 11, the highest doses of tandospirone tested increased pERK levels in the peripubertal PVN over saline-challenge. Again, within the dosing range tested, these responses approximate
the maximal response, although the additional data points at higher doses would better substantiate the respective $E_{\text{max}}$ values. These dose-response data indicate that (+)8-OH-DPAT (figure 10) is a more potent agonist than tandospirone on 5-HT$_{1A}$ receptor-mediated pERK responses in the PVN, with a more narrow dosing range ($\leq 1$ log unit) compared to tandospirone ($\geq 1$ log unit).

The two-way ANOVA for tandospirone-induced increases of pERK in the PVN indicated a significant main effect of acute challenge injection (saline or tandospirone) [$F_{1,69} = 19.66$, $p < 0.0001$] but no significant main effect of agonist dose [$F_{3,69} = 1.22$, $p = 0.3085$]. There was also no significant interaction between acute challenge injection and agonist dose [$F_{3,69} = 1.22$, $p = 0.3085$]. A Newman-Keuls’ test indicated that tandospirone increased levels of pERK in the PVN over saline-injected controls at 10 and 20 mg/kg ($p < 0.05$).

E. Optimal challenge dose of (+)8-OH-DPAT for future experiments. These results indicate that 0.20 mg/kg of (+)8-OH-DPAT can be used in subsequent studies to produce robust increases in 5-HT$_{1A}$ receptor-mediated plasma oxytocin and ACTH and levels of pERK in the peripubertal rat hypothalamic PVN.

F. Single injection of (+)8-OH-DPAT or tandospirone at any dose does not change testosterone levels in the peripubertal rat. As shown in figure 12, neither (+)8-OH-DPAT nor tandospirone had any effect on plasma levels of testosterone at any dose tested.
The two-way ANOVA for plasma testosterone levels indicated no significant main effect of acute challenge injection (saline, (+)8-OH-DPAT, or tandospirone) \([F_{2,91} = 0.07, p = 0.94]\) and no significant main effect of agonist dose \([F_{3,91} = 0.34, p = 0.79]\). There was also no significant interaction between acute challenge injection and agonist dose \([F_{6,91} = 0.19, p = 0.98]\). These data indicate that stage of sexual maturation was comparable between treatment groups and that the \textit{in vivo} challenge injections and doses did not alter the testosterone levels in these animals.
Figure 3. Time-course of (+)8-OH-DPAT and tandospirone-induced increases in plasma levels of oxytocin in the peripubertal rat. *, ** indicates significant difference (p < 0.05 and p < 0.01, respectively) from the respective saline-challenged group; #, ## indicates significant difference (p < 0.05 and p < 0.01, respectively) from the respective (+)8-OH-DPAT-challenged group; +, ++ indicates significant difference (p < 0.05 and p < 0.01, respectively) from corresponding response at 5 minute post-injection (two-way ANOVA and Newman-Keuls multiple-range test). N = 7-10 per group.
Figure 4. Time-course of (+)8-OH-DPAT and tandospirone-induced increases in plasma levels of ACTH in the peripubertal rat. ** indicates significant difference (p < 0.01) from the respective saline-challenged group; # indicates significant difference (p < 0.05) from the respective (+)8-OH-DPAT-challenged group; ++ indicates significant difference (p < 0.01) from corresponding response at 5 minute post-injection (two-way ANOVA and Newman-Keuls multiple-range test). N = 7-10 per group.
Figure 5. (A) Time-course of (+)8-OH-DPAT-induced elevations of pERK in peripubertal PVN. ** indicates significant difference (p < 0.01) from the saline-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). (B) Representative western blot of pERK (above) and ERK (below) from the PVN of rats treated with either saline or (+)8-OH-DPAT. N = 7-8 per group.
Figure 6. (A) Time-course of tandospirone-induced elevations of pERK in peripubertal PVN. * indicates significant difference (p < 0.05) from the saline-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). (B) Representative western blot of pERK (above) and ERK (below) from the PVN of rats treated with either saline or tandospirone. N = 6-8 per group.
Figure 7. Injection with either (+)8-OH-DPAT (0.20 mg/kg) or tandospirone (10 mg/kg) had no effect on plasma levels of testosterone in the peripubertal rat at any post-injection time point (two-way ANOVA). N = 3-9 per group.
Figure 8. Effects of different doses of (+)8-OH-DPAT and tandospirone on plasma levels of oxytocin in the peripubertal rat. ** indicates significant difference (p < 0.01) from the saline-challenged group; ++ indicates significant difference (p < 0.01) from corresponding lowest-dose challenge injection; ## indicates significant difference (p < 0.01) from the 0.01 mg/kg (+)8-OH-DPAT-challenged group; @ indicates significant difference (p < 0.05) from the 3 mg/kg tandospirone-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 7-11 per group.
**Figure 9.** Effects of different doses of (+)8-OH-DPAT and tandospirone on plasma levels of ACTH in the peripubertal rat. *,**, indicates significant difference (p < 0.05 and p < 0.01, respectively) from the saline-challenged group; ++ indicates significant difference (p < 0.01) from corresponding lowest-dose challenge injection; ## indicates significant difference (p < 0.01) from the 0.01 mg/kg (+)8-OH-DPAT-challenged group; ^^ indicates significant difference (p < 0.01) from the 0.02 mg/kg (+)8-OH-DPAT-challenged group; @ indicates significant difference (p < 0.05) from the 3 mg/kg tandospirone-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 7-10 per group.
Figure 10. (A) Effects of different doses of (+)8-OH-DPAT on levels of pERK in peripubertal PVN. *, ** indicates significant difference (p < 0.05 and p < 0.01, respectively) from the saline-challenged group; +, ++ indicates significant difference (p < 0.05 and p < 0.01 respectively) from the 0.04 mg/kg (+)8-OH-DPAT-challenged group; #, ## indicates significant difference (p < 0.05 and p < 0.01 respectively) from the 0.01 mg/kg (+)8-OH-DPAT-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). (B) Representative western blot of pERK (above) and ERK (below) from the PVN of rats treated with either saline or (+)8-OH-DPAT. N = 6-12 per group.
Figure 11. (A) Effects of different doses of tandospirone on levels of pERK in peripubertal PVN. * indicates significant difference (p < 0.05) from the saline-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). (B) Representative western blot of pERK (above) and ERK (below) from the PVN of rats treated with either saline or tandospirone. N = 4-12 per group.
Figure 12. Injection with different doses of either (+)8-OH-DPAT or tandospirone had no effect on plasma levels of testosterone in the peripubertal rat (two-way ANOVA). N = 4-10 per group.
DISCUSSION

Several immunohistochemical studies have determined that 5-HT$_{1A}$ receptors are expressed on many types of neuroendocrine-containing cells (e.g., neuropeptide Y-, proopiomelanocortin-, orexin-containing, etc.) throughout the hypothalamus (Collin et al., 2002; Aznar et al., 2003; Muraki et al., 2004; Marvin et al., 2010). 5-HT$_{1A}$ receptors have also been localized to glial cells in other brain regions (Whitaker-Azmitia et al., 1993; Azmitia et al., 1996; Paterson et al., 2004). In the adult PVN, oxytocin-containing and CRH-containing cells express 5-HT$_{1A}$ receptors (Zhang et al., 2004). Stimulation of these receptors with different classes of 5-HT$_{1A}$ receptor agonists such as (+)8-OH-DPAT (an aminotetralin) or tandospirone (an azapirone) can increase plasma levels of oxytocin and ACTH hormones (Bagdy and Kalogeras, 1993; Gilbert et al., 1988; Vicentic et al., 1998). In the adult PVN, these agonists also concomitantly activate ERK (Chen et al., 2002; Crane et al., 2007; Sullivan et al., 2005). The data from the present studies are the first to demonstrate in the peripubertal PVN: (1) that both (+)8-OH-DPAT and tandospirone increased plasma levels of oxytocin and ACTH and increased levels of pERK (indicating that 5-HT$_{1A}$ receptors also can concomitantly activate multiple responses prior to sexual maturation) and (2) the pharmacodynamic properties of these different classes of 5-HT$_{1A}$ receptor agonists at each of the respective signaling responses. These studies indicate that, although 5-HT$_{1A}$ receptors activate neuroendocrine responses and ERK in both the adult and
peripubertal PVN, there are some aspects of 5-HT$_{1A}$ receptor-mediated activation of ERK that differ between these age groups that may play a role in acute drug responsivity or to long term drug-induced neuroadaptations.

One notable difference observed between adult and peripubertal 5-HT$_{1A}$ receptor signaling is in the duration of MAPK responses. In the adult PVN, 5-HT$_{1A}$ receptors activate ERK in a rapid and transient manner, as the aminotetralin agonist (+)8-OH-DPAT significantly elevates levels of activated ERK at 5 minutes but returns to basal levels by 15 minutes post-injection (Crane et al., 2007). The current studies have demonstrated that 5-HT$_{1A}$ receptor-stimulated pERK in the peripubertal PVN is also rapid, but the response is more prolonged than in adults. This prolonged activation of ERK occurs following 5-HT$_{1A}$ receptor activation by either class of agonist, since both (+)8-OH-DPAT and tandospirone injections resulted in elevations in pERK lasting up to at least 30 minutes post-injection. In a variety of mammalian cell lines, the duration of ERK signaling is associated with distinct cell behavior (Traverse et al., 1992; Nguyen et al., 1993; Mansour et al., 1994; Sharp et al., 1997; Whalen et al., 1997; Murphy et al., 2002; Murphy et al., 2004; Sinnett-Smith et al., 2004). For example, prolonged but not transient ERK activation was associated with (1) neuronal differentiation in PC-12 cells (Traverse et al., 1992), (2) nuclear translocation of ERK in PC12 cells (Nguyen et al., 1993), and (3) c-fos accumulation and DNA synthesis in Swiss 3T3 cells (Sinnett-Smith et al., 2004). Therefore, developmental differences
in the longevity of ERK activation may impact a variety of functions under the control of ERK (e.g., cell survival, gene expression, learning, memory, and receptor desensitization) (Marshall 1995; Pouysségur and Lenormand 2003; Trincavelli et al., 2002). Considering that ERK signaling has also recently been implicated in stress behaviors and the effects of antidepressants in animal models (Duman et al., 2007; Duric et al., 2010; Gourley et al., 2008; Qi et al., 2006; Qi et al., 2008; Qi et al., 2009; Shen et al., 2004), a prolonged ERK response in response to 5-HT1A receptor activation in the peripubertal brain could possibly influence the mechanism of action of antidepressants in this age group. For example, Duric et al., (2010) determined that overexpression (by viral vectors or induction via chronic stress) of MAP kinase phosphatase (MKP-1) in the hippocampus results in depressive behavior in a rodent model, presumably due to the more rapid dephosphorylation of ERK and termination of its signal. Further studies are required to determine if the temporal differences between peripubertal and adult are due to mechanisms involving ERK activation or phosphatase activity.

With respect to comparative studies using an aminotetralin versus azapirone 5-HT1A receptor agonists, (+)8-OH-DPAT is classified a “full” agonist and tandospirone is considered a “partial” agonist at post-synaptic 5-HT1A receptors. In rat hippocampal homogenates, tandospirone partially inhibits forskolin-induced adenylyl cyclase activity (Hamik et al., 1990; Rabin and Winter, 1993).
Buspirone, another azapirone, also acts as a partial agonist on neuronal hyperpolarization in vitro (Van den Hooff and Galvan, 1992). However, in the peripubertal rat, maximally effective doses of the aminotetralin ((+)-8-OH-DPAT) or azapirone (tandospirone) agonists produce comparable increases in each of the respective 5-HT$_{1A}$ receptor-mediated responses. The current studies suggest that tandospirone can act as a full agonist on 5-HT$_{1A}$ receptor-mediated oxytocin and ACTH plasma hormone responses and activation of ERK in the PVN. This finding is not unprecedented, given that tandospirone has been shown to act as a full agonist in inducing 5-HT$_{1A}$ receptor-mediated hypothermia in rats (Millan et al., 1993).

Given the multiplicity of 5-HT$_{1A}$ receptor signaling responses in peripubertal PVN, these studies also sought to determine the existence of “biased agonism” of 5-HT$_{1A}$ receptor-mediated responses in the peripubertal PVN. This phenomenon (which expands upon the two-state model of receptor activation) states that, whereas one class of receptor agonists stabilize the active conformation (\(R \rightarrow R^*1\)) that favors the activation of pathway 1, another class of receptor agonists stabilize an alternate active receptor conformation (\(R \rightarrow R^*2\)) that favors activation of pathway 2 (Rozengurt 2007). This phenomenon is observed in other receptor systems in vitro (Berg et al., 1998; Galandrin and Bouvier, 2006; Kenakin 1995). Berg et al. demonstrated that a piperazine derivative preferentially activates the 5-HT$_{2C}$ receptor-mediated phospholipase C pathway, whereas a
member of the ergoline family preferentially activates the 5-HT$_{2C}$ receptor-mediated phospholipase A$_2$ pathway (Berg et al., 1998). A literature search revealed that no in vivo studies have investigated this phenomenon of “biased agonism” for 5-HT$_{1A}$ receptor-mediated signaling pathways. Our results suggest that 5-HT$_{1A}$ receptor responses in the peripubertal PVN are not subject to “biased agonism” with respect to azapirone versus aminotetralin agonists since each agonist elicited similar maximal responses in 5-HT$_{1A}$ receptor-mediated oxytocin, ACTH, and pERK responses. However, this finding must be considered preliminary, considering that it has not been determined whether these receptor responses in the peripubertal PVN are activated as a result of dependent vs. multiple independent pathways mediated by separate subsets of second messenger proteins (e.g., Go proteins). The role of pertussis toxin-sensitive Go proteins will be investigated in Chapter 4.

Although both agonists tested in the current experiments had similar maximal efficacies for 5-HT$_{1A}$ receptor-mediated neuroendocrine responses, (+)8-OH-DPAT is generally more potent than tandospirone for 5-HT$_{1A}$ receptor-mediated oxytocin and pERK responses. The current data also indicate that (+)8-OH-DPAT generally has a narrower dosing range for 5-HT$_{1A}$ receptor-mediated increases in plasma levels of oxytocin than tandospirone. Consequently, (+)8-OH-DPAT may be able to occupy a fewer number of receptors to induce 5-HT$_{1A}$ receptor-mediated responses than tandospirone, suggesting that it more
efficiently couples receptor occupation to response. A more likely explanation is that (+)8-OH-DPAT may become more readily bioavailable compared to tandospirone. Bioavailability is a pharmacokinetic property that refers to the percentage of unchanged drug that reaches systemic circulation following any route of administration (Holford 2004). Therefore, depending on drug class, a greater percentage of active drug may reach the brain. Although it was been determined that within 15 minutes of subcutaneous administration of (+)8-OH-DPAT, approximately 0.1% of the active drug dose was detected in rat hypothalamus (Yu and Lewander 1997), comparable studies have not examined hypothalamic concentrations after acute subcutaneous tandospirone injection in rats. However, about 0.04% of tandospirone was detected in cortex within 30 minutes of intraperitoneal injection (1-20 mg/kg) in mice (Miller et al., 1992). Similarly, the clinically prescribed azapirone drug buspirone has been reported to have a very low bioavailability (Mahmood and Sahajwalla, 1999). Also of note, lower doses of tandospirone increased maximal plasma levels of ACTH, but not oxytocin or increased pERK in PVN. This is likely due to the 5-HT_{1A} receptor-mediated activation of the sequential pathway (e.g. CRH → ACTH) mediated oxytocin plasma hormone responses. Although not as obvious, lower doses of (+)8-OH-DPAT also significantly elevated plasma ACTH responses over saline, but had no significant effect on plasma levels of oxytocin and pERK responses in PVN.
Lastly, although 5-HT$_{1A}$ receptors have been localized to both oxytocin-containing and CRH-containing cells in the rat PVN (Zhang et al., 2004), it is unknown whether each of these respective receptor populations are responsible for mediating both plasma hormone responses and increases in pERK measured at the level of the whole PVN. Studies in other receptor systems (α1 adrenergic) have shown that receptor-stimulated pERK in the PVN can be observed in CRH-containing cells (Khan et al., 2007). However, although stimulation with prolactin or IL-1β increases levels of pERK in the hypothalamus, there is little localization of pERK to oxytocin-containing cells (Blume et al., 2009; Wang et al., 2006). Cellular localization of 5-HT$_{1A}$ receptor-mediated pERK will be further examined in Chapter 5 of this dissertation.

From the current experiments, we have determined that a dose of 0.20 mg/kg (+)8-OH-DPAT and a sacrifice time of 10 minutes post-injection will be used in future experiments examining 5-HT$_{1A}$ receptor signaling pathways following acute receptor stimulation since it produces robust increases in each of the respective endpoints and is devoid of altering testosterone levels which could complicate any interpretation of the data. Since 0.20 mg/kg of (+)8-OH-DPAT elicits the maximal 5-HT$_{1A}$ receptor-mediated oxytocin and ACTH responses, the dose of (+)8-OH-DPAT may not be sensitive to detect desensitization of 5-HT$_{1A}$ receptor-mediated hormone responses due to chronic treatment with antidepressants or other drugs.
CHAPTER 4

SEROTONIN 1A RECEPTOR-ASSOCIATED Ga PROTEIN SUBTYPES IN THE PERIPUBERTAL RAT HYPOTAHLMIC PARAVENTRICULAR NUCLEUS: UNIQUE ASPECTS OF MODIFICATION AND REGULATION.

ABSTRACT

In adult rats, injection with Bordetella pertussis toxin (PTX) into the paraventricular nucleus (PVN) reduces levels of Ga1, Ga2, Ga3, and Gao proteins and blocks serotonin 1A (5-HT1A) receptor-mediated extracellular signal-regulated kinase (ERK) activation but has no effect on Gaz proteins or 5-HT1A receptor-mediated plasma oxytocin or ACTH responses (Garcia et al., 2006). These data indicate that 5-HT1A receptors in the adult PVN activate separate and distinct signal transduction pathways. Consistent with these data, chronic treatment of adult rats with fluoxetine, a selective serotonin reuptake inhibitor (SSRI), selectively reduces hypothalamic levels of Gaz and desensitizes 5-HT1A receptor-mediated neuroendocrine responses but does not reduce any of the other Ga proteins or affect 5-HT1A receptor-mediated increases in pERK. In contrast, chronic fluoxetine treatment of peripubertal rats desensitizes 5-HT1A receptor-mediated in the absence in reductions of Gaz proteins (Chen et al., 2006), suggesting that, prior to maturation, Gaz is not the exclusive Ga subtype mediating hormone responses as it is in adults. Given that 5-HT1A receptor-
mediated activation of ERK was not determined in that previous study, the Ga protein subtype(s) specificity for the respective 5-HT$_{1A}$ receptor-mediated responses prior to sexual maturation has not been identified. The present studies examined 5-HT$_{1A}$ receptor-associated Ga proteins in the peripubertal rat hypothalamic PVN with respect to: (1) levels of the respective membrane-associated Ga proteins at peripubertal versus younger (prepubertal) and adult ages, (2) the role of PTX-sensitive and PTX-insensitive Ga proteins on 5-HT$_{1A}$ receptor-mediated increases in oxytocin and ACTH and increases of pERK in the PVN, and (3) the effects of chronic fluoxetine treatment (7 or 14 days) in regulating Ga protein subtypes and 5-HT$_{1A}$ receptor-mediated activation of ERK in PVN. The present studies indicate that, in peripubertal rats: (1) Gao is the only Ga protein subject to developmental regulation (i.e., Gao levels increased with age) whereas Ga1, Ga2, Ga3, and Gaoz were present at comparable levels in PVN, hippocampus, and frontal cortex of the prepubertal, peripubertal, and adult brain, (2) PTX treatment reduced levels of membrane-associated Ga1 and Ga2, markedly increased levels of Gao, but did not alter Ga3 or Gaoz in the peripubertal hypothalamus, (3) PTX-induced reductions in Ga1 and Ga2 proteins did not affect 5-HT$_{1A}$ receptor-mediated oxytocin or ACTH responses, suggesting a role for Ga3, Gao, and/or Gaoz proteins in 5-HT$_{1A}$ receptor-mediated signaling, (4) PTX increased basal pERK levels, suggesting an increased constitutive activity of pERK consistent with increases in Gao, (5) chronic
fluoxetine did not reduce levels either cytosol- or membrane-associated Gαz or any other Gα protein subtype, suggesting a novel mechanism responsible for fluoxetine-induced desensitization of 5-HT_{1A} receptor-mediated neuroendocrine responses, and (6) chronic fluoxetine did alter basal levels of pERK but did not desensitize 5-HT_{1A} receptor-mediated increases in pERK in the PVN. In summary, the data from the current studies identify a different profile of Gα protein sensitivity to PTX and chronic fluoxetine in the peripubertal rat than previously established in the adult rat. These aspects of Gα protein subtypes not observed in adults but unique to the peripubertal brain, precluded any effective use of PTX or fluoxetine to differentiate the role of Gai/o versus Gαz proteins in the respective roles of these subtypes in 5-HT_{1A} receptor-mediated neuroendocrine versus kinase responses.

INTRODUCTION

5-HT_{1A} receptors can couple to multiple signal transduction pathways (Raymond et al., 2001) via different subsets of PTX-sensitive Gα proteins (Gai1, Gai2, Gai3, and Gao) or PTX-insensitive Gαz proteins (Mannoury la Cour et al., 2006; Raymond et al., 1992; Serres et al., 2000a). The in vivo signal transduction pathways mediated by these two subsets of proteins in hypothalamic PVN and their regulation by different drug treatments have thus far only been investigated in adult rats. In the adult PVN, Gαz proteins (insensitive to pertussis toxin) mediate the 5-HT_{1A} receptor-induced increases in plasma ACTH
and oxytocin (Serres et al., 2000a). In contrast, one or more subtypes of Ga/i/o proteins mediate the 5-HT$_{1A}$ receptor-induced increase in activated ERK in hypothalamus, since intra-PVN injection with PTX completely inhibits 5-HT$_{1A}$ receptor activation of ERK and reduces Ga1, Ga2, Ga3, and Ga0 proteins. In contrast, intra-PVN injection with PTX does not alter 5-HT$_{1A}$ receptor-mediated stimulation of neuroendocrine responses or reduce levels of GaZ proteins (Garcia et al., 2006). Thus, in the adult rat, PTX provides a useful tool to differentiate signaling by these respective subsets of G proteins (i.e., PTX-sensitive or PTX-insensitive). These 5-HT$_{1A}$ receptor-mediated pathways in the adult PVN were also differentially regulated by chronic drug treatment: fluoxetine consistently produced reductions in GaZ and desensitized 5-HT$_{1A}$ receptor-mediated neuroendocrine responses but did not alter 5-HT$_{1A}$ receptor-mediated activation of ERK or levels of Ga/i/o proteins in adult rats (Jia et al., 2006). Therefore, it is likely that Ga/i/o and GaZ proteins mediate signaling of separate and distinct pathways following acute activation of 5-HT$_{1A}$ receptors in the adult rat PVN.

A preliminary study from our laboratory suggested that the signaling of 5-HT$_{1A}$ receptor-mediated responses in peripubertal animals occurred via mechanism(s) that differ from adult animals. In peripubertal rats, chronic fluoxetine treatment produced desensitization of 5-HT$_{1A}$ receptor-mediated hormone responses and also decreased levels of hypothalamic Ga3 and GaO proteins but had no effect on hypothalamic levels of GaZ proteins (Chen et al.,
This differential profile of reductions in $G\alpha$ protein subtypes and the lack of reduction in $G_{az}$ despite a desensitization of $5\text{-HT}_{1A}$ receptor-mediated hormone responses suggest that, prior to sexual maturation, other $G\alpha$ proteins or mediators are responsible for the desensitization of hormone responses. Consequently, reductions in $G_{az}$ are not responsible for desensitization of hormone responses.

Differences observed in fluoxetine-induced reductions in $G\alpha$ proteins in peripubertal vs. adult hypothalamus may be simply due to developmental differences in the levels of $G\alpha$ proteins, which could affect the subtype associated with hormone stimulation. $G\alpha$ protein levels vary in early postnatal development in several brain regions. For example, $Gai1$, $Gai2$, and $Gao$ proteins increase between postnatal day 1 and 25 in the cortex, thalamus, and hippocampus, where $Gai3$ proteins decrease in these regions over the same time period (Ihnatovych et al., 2002). However, no studies have determined the developmental regulation of $G_{az}$ in any brain region, or any $G\alpha$ protein subset in the hypothalamus. In addition, no studies have examined the regional distribution of $G\alpha$ proteins in brain during peripubertal development, an important period for brain growth and remodeling. Any developmental change in the availability of $5\text{-HT}_{1A}$ receptor-coupled $G\alpha$ proteins may impact the role that these $G\alpha$ proteins play in $5\text{-HT}_{1A}$ receptor-mediated signaling of neuroendocrine vs. kinase responses.
Fluoxetine is one of two SSRIs currently approved for clinical use for the treatment of depressive disorders in children and adolescents (National Institutes of Health, 2010). Fluoxetine is one of the most commonly used SSRI in preclinical studies, particularly those involving peripubertal animal models (Oh et al., 2009; Iniguez et al., 2010).

The objectives of the experiments described in this chapter were to determine: (1) if the relative levels of Gα protein subtypes in different regions in the peripubertal brain vs. younger (prepubertal) and adult brains, (2) the role of PTX-sensitive Gα proteins in 5-HT1A receptor-mediated neuroendocrine responses and phosphorylation of ERK in the peripubertal PVN using an intra-PVN injection of PTX, and (3) the regulation Gα proteins and 5-HT1A receptor-mediated activation of ERK in the peripubertal PVN upon chronic (7 and 14 day) fluoxetine treatment.

**MATERIALS AND EXPERIMENTAL PROCEDURES (for detailed methods, see Chapter 7)**

**Animals.** Prepubertal, peripubertal and adult male Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN). The determination of post-natal day zero (day of birth) was carried out by the supplier.

**Drugs.** Pertussis toxin (PTX; *Bordetella pertussis*) was purchased from EMD Chemicals, Gibbstown, NJ (catalog number 516560), and was diluted to a concentration of 1 μg/μl with ddH2O. Fluoxetine hydrochloride (FLX) was
purchased from LKT Laboratories, Inc., St. Paul, MN (catalog number F4780), and was dissolved in saline (0.9% NaCl) to 10 mg/kg and was injected at a volume of 2 ml/kg. (R)-(+) -8-Hydroxy-2-(di-n-propylamino)tetralin hydrobromide [(+)8-OH-DPAT] was purchased from Tocris Cookson Inc., Ellisville, MO (catalog number 1080) and was dissolved in saline (0.9% NaCl) to 0.20 mg/ml and was injected at a volume of 1 ml/kg. All drugs were freshly prepared immediately prior to the injections.

**Experiment 1: Age-dependent brain levels of Gα protein subtypes that mediate signaling of 5-HT1A receptors.** Male Sprague-Dawley rats at three different ages (prepubertal, peripubertal, and adult) were handled for several days to reduce the stress of handling upon sacrifice. Each rat was sacrificed by decapitation on postnatal day (PD) 28 (prepubertal), PD 42 (peripubertal), or PD 70 (adult), and the brains were quickly removed and frozen on dry ice for western blot determination of Gα protein levels in different brain regions. The trunk blood was collected in tubes and prepared for subsequent determination of plasma levels of testosterone.

**Experiment 2: Pertussis toxin inactivation of Gα proteins in the peripubertal rat hypothalamic PVN.** Cannula implantation and administration of PTX was performed according to procedures previously established in adult rats (Garcia et al., 2006), and detailed in Chapter 7. Seventy-two hours after intracerebral injection of vehicle (100 mM sodium phosphate + 500 mM NaCl, 0.5 μl/side) or
PTX (1 μg/μl, 0.5 μl/side), the rats were given a systemic challenge injection of saline (1 ml/kg) or (+)8-OH-DPAT (0.20 mg/kg) and were sacrificed by decapitation 15 minutes later (postnatal day 42). The brains were carefully removed from the skull within one minute of the decapitation and were quickly frozen by submersion in chilled 2-methylbutane (-35°C) for 1 minute and then subsequently kept in dry ice for an additional 10 minutes. Each brain was then securely wrapped and stored at -80°C prior to dissection for Western blot studies for the determination of Ga protein levels and pERK/ERK levels in the PVN. The trunk blood was collected in tubes and prepared for subsequent determination of plasma levels of oxytocin, ACTH, and testosterone.

**Experiment 3: Determination of fluoxetine-induced effects on 5-HT₁A receptor-associated Ga proteins and 5-HT₁A receptor-mediated increases of pERK in the peripubertal PVN.** Rats were weighed on a digital scale daily and were subsequently given an i.p. injection of saline (2 ml/kg) or fluoxetine (10 mg/kg), once per day for either 7 (PD 35-41) or 14 days (PD 28-41). This fluoxetine dosing regimen has been previously shown to desensitize 5-HT₁A receptor-mediated oxytocin and ACTH responses and to inhibit weight gain in peripubertal rats (Chen et al., 2006). Eighteen hours after the last injection, the rats (postnatal day 42) were given a systemic challenge injection of saline (1 ml/kg) or (+)8-OH-DPAT (0.20 mg/kg) and were sacrificed by decapitation 10 minutes later. The brains were carefully removed from the skull within one
minute of the decapitation and were quickly frozen by submersion in chilled 2-
methylbutane (-35°C) for 1 minute and then subsequently kept in dry ice for an 
additional 10 minutes. Each brain was then securely wrapped and stored at 
-80°C prior to dissection for Western blot studies for the determination of Ga 
protein levels and pERK/ERK levels in the PVN. The trunk blood was collected 
in tubes and prepared for subsequent determination of plasma levels of oxytocin, 
ACTH, and testosterone.

RESULTS

1. **Experiment 1: Determination of relative levels of Ga proteins in the PVN and 
other brain regions at different developmental stages.** The objective of this 
experiment was to determine the levels of the respective Ga proteins at 
peripubertal versus younger (prepubertal) and adult ages in various brain 
regions. The body weights and plasma testosterone levels were also measured 
for each rat.

A. **Average body weight of prepubertal, peripubertal, and adult rats.** Rats 
gained weight with age. Prepubertal rats weighed, on average, 76.96 ± 0.76 g, 
peripubertal rats weighed about twice as much as prepubertal rats (164.76 ± 0.91 
g), and adult rats weighed about four times as much as prepubertal rats (325.79 ± 
1.36 g) (figure 13).

The one-way ANOVA for body weight indicated a significant main effect of 
age \([F_{2,29} = 14608.16, \ p < 0.0001]\). A Newman-Keuls’ test indicated that
peripubertal rats weighed significantly more than prepubertal rats (p < 0.01) and that adults weighed significantly more than both prepubertal and peripubertal rats (p < 0.01).

B. Plasma testosterone levels of prepubertal, peripubertal, and adult rats. Plasma levels of testosterone increased with age. Prepubertal rats had an average plasma testosterone level of 13.56 ± 2.04 ng/dl, peripubertal rats had about 4.5 times higher levels than prepubertal rats (61.30 ± 9.73 ng/dl), and adult rats had about 17.5 times higher levels than prepubertal rats (236.97 ± 25.80 ng/dl) (figure 14).

The one-way ANOVA for plasma testosterone levels indicated a significant main effect of age [F2,29 = 54.31, p < 0.0001]. A Newman-Keuls’ test indicated that peripubertal rats had greater levels of plasma testosterone than prepubertal rats (p < 0.05) and that adults had greater levels of plasma testosterone than both prepubertal and peripubertal rats (p < 0.01).

C. Ga protein levels in the PVN of prepubertal, peripubertal, and adult rats. In the PVN, Gαo proteins, but not Gαi1, Gαi2, Gαi3, or Gαz proteins, increased with age. Gαo proteins were present in the prepubertal PVN at about 40% adult levels and in the prepubertal PVN at about 68% adult levels (figure 15, table 2).

The one-way ANOVA for Gαi1 (figure 15A) indicated no significant main effect of age [F2,23 = 0.12, p = 0.8856]. The one-way ANOVA for Gαi2 (figure 15B) indicated no significant main effect of age [F2,22 = 0.94, p = 0.4082]. The one-way
ANOVA for Gαi3 (figure 15C) indicated no significant main effect of age \([F_{2,19} = 0.06, p = 0.9407]\). The one-way ANOVA for Gαz (figure 15D) indicated no significant main effect of age \([F_{2,19} = 0.73, p = 0.4969]\). The one-way ANOVA for Gαo (figure 15E) indicated a significant main effect of age \([F_{2,20} = 5.53, p = 0.0123]\). A Newman-Keuls’ test indicated that the adults had significantly higher levels of Gαo proteins than prepubertal rats \((p < 0.01)\).

D. Gα protein levels in the hippocampus of prepubertal, peripubertal, and adults rats. In the hippocampus, Gαo proteins, but not Gαi1, Gαi2, Gαi3, or Gαz proteins, increased with age. Gαo proteins were present in the prepubertal hippocampus at about 26% adult levels and in the prepubertal PVN at about 58% adult levels (table 3).

The one-way ANOVA for Gαi1 indicated no significant main effect of age \([F_{2,21} = 1.55, p = 0.237]\). The one-way ANOVA for Gαi2 indicated no significant main effect of age \([F_{2,19} = 0.25, p = 0.7805]\). The one-way ANOVA for Gαi3 indicated no significant main effect of age \([F_{2,21} = 0.06, p = 0.9463]\). The one-way ANOVA for Gαo indicated a significant main effect of age \([F_{2,22} = 14.52, p = 0.0001]\). A Newman-Keuls’ test indicated that peripubertal rats had significantly more Gαo protein levels in the hippocampus than prepubertal rats \((p < 0.05)\) and that adults had significantly higher levels of Gαo proteins than prepubertal rats and peripubertal rats \((p < 0.01)\). The one-way ANOVA for Gαz indicated no significant main effect of age \([F_{2,22} = 0.16, p = 0.8506]\).
E. **Gα protein levels in the frontal cortex of prepubertal, peripubertal, and adult rats.** In the frontal cortex, Gαo proteins, but not Gα1, Gα2, Gα3, or Gαz proteins, increased with age. Gαo proteins were present in the prepubertal frontal cortex at about 45% adult levels and in the prepubertal PVN at about 62% adult levels (table 4).

The one-way ANOVA for Gα1 indicated no significant main effect of age \[ F_{2,20} = 0.15, p = 0.8652 \]. The one-way ANOVA for Gα2 indicated no significant main effect of age \[ F_{2,21} = 0.47, p = 0.6326 \]. The one-way ANOVA for Gα3 indicated no significant main effect of age \[ F_{2,22} = 0.26, p = 0.7728 \]. The one-way ANOVA for Gαo indicated a significant main effect of age \[ F_{2,22} = 43.66, p < 0.0001 \]. A Newman-Keuls’ test indicated that peripubertal rats had significantly more Gαo protein levels in the frontal cortex than prepubertal rats \( p < 0.01 \) and that adults had significantly higher levels of Gαo proteins than prepubertal rats and peripubertal rats \( p < 0.01 \). The one-way ANOVA for Gαz indicated no significant main effect of age \[ F_{2,20} = 0.44, p = 0.6522 \].

2. **Experiment 2: Determination of the role of PTX-sensitive Gai/o proteins in 5-HT$_{1A}$ receptor-mediated signaling in peripubertal hypothalamic PVN.** The objective of this experiment was to use pertussis toxin (PTX) as previously demonstrated in adult rats (Garcia et al., 2006) to ADP ribosylate and functionally inactivate Gai/o proteins to determine if this subset of proteins plays a role in 5-HT$_{1A}$ receptor-induced increases of plasma levels of oxytocin.
and ACTH and increases in pERK in the peripubertal PVN.

A. Effects of PTX treatment on hypothalamic levels of Ga proteins. Intra-PVN injection of PTX in peripubertal rats reduced hypothalamic levels of Gαi1 and Gαi2 (by 36% and 47%, respectively), increased levels of Gαo (by 96%), and had no effect on the levels of Gαi3 and Gαz (figure 16).

The Student’s t-test indicated that PTX treatment significantly decreased Gαi1 proteins [t value = 4.03, p = 0.0007]. The Student’s t-test indicated that PTX treatment significantly decreased Gαi2 proteins [t value = 3.49, p = 0.0026]. The Student’s t-test for Gαi3 indicated no effect of PTX treatment [t value = 0.98, p = 3411]. The Student’s t-test indicated that PTX treatment significantly increased Gαo proteins [t value = -3.86, p = 0.0012]. The Student’s t-test for Gαz indicated no effect of PTX treatment [t value = -1.06, p = 0.3030].

B. Effects of PTX treatment on 5-HT1A receptor-mediated increases in plasma levels of oxytocin in the peripubertal rat. (+)8-OH-DPAT increased plasma levels of oxytocin over 20 times that over saline injection. Intra-PVN injection with PTX in peripubertal rats did not significantly reduce (+)8-OH-DPAT-induced increases in plasma oxytocin levels over saline injection (figure 17).

The two-way ANOVA for oxytocin indicated no main effect of PTX treatment [F1,64 = 1.38, p = 0.2448] but a significant main effect of challenge injection [F1,64 = 452.23, p < 0.0001]. There was no significant interaction between treatment and challenge injection [F1,64 = 2.47, p = 0.121]. A Newman-Keuls’ test indicated that
(+)-8-OH-DPAT challenge significantly elevated plasma oxytocin levels over saline-challenged in both vehicle-treated and PTX-treated groups (p < 0.01).

C. **Effects of PTX treatment on 5-HT\textsubscript{1A} receptor-mediated increases in plasma levels of ACTH in the peripubertal rat.** (+)-8-OH-DPAT increased plasma levels of ACTH roughly 10 times that over saline injection. Intra-PVN injection with PTX in peripubertal rats slightly increased basal levels of ACTH but did not significantly reduce the (+)-8-OH-DPAT-induced increase in ACTH over saline injection (figure 18).

The two-way ANOVA for ACTH indicated no main effect of PTX treatment \([F_{1,65} = 1.00, p = 0.3207]\) but a significant main effect of challenge injection \([F_{1,65} = 672.81, p < 0.0001]\). There was no significant interaction between treatment and challenge injection \([F_{1,65} = 3.76, p = 0.0571]\). A Newman-Keuls’ test indicated that (+)-8-OH-DPAT challenge significantly elevated plasma ACTH levels over saline-challenged in both vehicle-treated and PTX-treated groups (p < 0.01).

D. **Effects of PTX treatment on basal and 5-HT\textsubscript{1A} receptor-mediated increases in levels of pERK in PVN.** Challenge with (+)-8-OH-DPAT increased pERK in the PVN in the vehicle-treated group about 97% over saline challenge. Intra-PVN injection with PTX in peripubertal rats increased basal (saline) levels of phosphorylated ERK in the PVN increased about 95%. (+)-8-OH-DPAT mediated a further increase of only 40% over its respective basal (figure 19). Therefore, injection with PTX either attenuated 5-HT\textsubscript{1A} receptor-mediated phosphorylation
of ERK in the PVN or increased basal levels of pERK to a degree that further receptor stimulation did not induce a statistically significant change over basal.

The two-way ANOVA for levels of phosphorylated ERK in the PVN indicated a main effect of PTX treatment \[F_{1,28} = 4.36, p = 0.0471\] and a significant main effect of challenge injection \[F_{1,28} = 4.51, p = 0.0438\]. There was no significant interaction between treatment and challenge injection \[F_{1,28} = 0.06, p = 0.8148\]. Although the basal levels of phosphorylated ERK in the PVN increased by about 95% with PTX treatment, this did not reach statistical significance, nor did 5-HT$_{1A}$ receptor-stimulated elevations of pERK in vehicle-treated (↑97% over respective basal) and PTX-treated (↑40% over respective basal) groups.

E. Effects of PTX on plasma levels of testosterone in the peripubertal rat. Intra-PVN injection with PTX or challenge injection with (+)8-OH-DPAT in peripubertal rats had no effect on the plasma levels of testosterone. The average plasma testosterone levels of all treatment groups ranged between 15-28 ng/dl (figure 20).

The two-way ANOVA for plasma testosterone levels indicated no significant main effect of PTX treatment \[F_{1,37} = 1.11, p = 0.3005\] and no significant main effect of challenge injection \[F_{1,37} = 1.65, p = 0.2073\]. There was also no significant interaction between PTX treatment and challenge injection \[F_{1,37} = 0.13, p = 0.7165\].
3. **Experiment 3: Determination of SSRI-induced regulation of 5-HT$_{1A}$ receptor-coupled $\text{G}_\alpha$ proteins and 5-HT$_{1A}$ receptor-mediated ERK signaling in peripubertal hypothalamic PVN.** The objective of this experiment was to use chronic fluoxetine (FLX) injections (once per day for 7 or 14 days) to selectively reduce $\text{G}_\alpha i3$ and $\text{G}_\alpha o$ proteins (as previously observed in Chen et al., 2006) to determine if these $\text{G}_\alpha$ proteins play a role in 5-HT$_{1A}$ receptor-mediated pERK responses in the peripubertal PVN. In the peripubertal rat, 14 days of chronic FLX induced desensitization of 5-HT$_{1A}$ receptor-mediated increases of plasma levels of both oxytocin and ACTH in response to challenge with a dose of (+)8-OH-DPAT (0.04 mg/kg) that is submaximal for 5-HT$_{1A}$ receptor-mediated neuroendocrine responses (Chen et al., 2006). In the current experiment, we used a higher challenge dose of (+)8-OH-DPAT (0.20 mg/kg) in order to measure pERK responses in the PVN. However, this dose is near the maximal dose necessary to elicit neuroendocrine responses (see chapter 3). Therefore, we did not expect to observe a blunted neuroendocrine response in response to fluoxetine treatment at this dose of (+)8-OH-DPAT.

A. **Effect of chronic fluoxetine treatment of peripubertal rats on body weight gain.** Rats from all chronic treatment groups gained weight with age. Chronic fluoxetine (7 and 14 days) treatment attenuated body weight gain in peripubertal rats, with significant effects emerging around treatment day 4 (figure 21).
For 7 days of treatment, a repeated measures ANOVA indicated that there was no significant main effect of treatment \([F_{1,407} = 0.42, p = 0.5188]\) but a significant main effect of 7 days treatment time \([F_{7,407} = 3433.34, p < 0.0001]\). There was also a significant interaction between treatment group and treatment time \([F_{7,407} = 12.71, p < 0.0001]\). A Newman-Keuls’ test indicated that fluoxetine significantly attenuated weight gain as compared to saline-treated rats on injection days 4 \((p < 0.05)\) and 5-7 \((p < 0.01)\).

For 14 days of treatment, a repeated measures ANOVA indicated that there was no significant main effect of treatment \([F_{1,719} = 2.02, p = 0.1624]\) but there was a significant main effect of 14 days treatment time \([F_{14,719} = 4642.33, p < 0.0001]\). There was also a significant interaction between treatment group and treatment time \([F_{14,719} = 4.06, p < 0.0001]\). A Newman-Keuls’ test indicated that fluoxetine significantly attenuated weight gain as compared to saline-treated rats on injection days 5 \((p < 0.05)\) and 7-14 \((p < 0.01)\).

B. Effect of chronic fluoxetine treatment of peripubertal rats on membrane-associated and cytosol-associated \(\alpha\) proteins in PVN. In peripubertal rats, chronic fluoxetine treatment for either 7 days (figure 22) or 14 days (figure 23) did not reduce membrane-or cytosol-associated \(\alpha1, \alpha2, \alpha3, \alpha0,\) or \(\alpha z\) protein levels in the PVN.

The Student’s t-test for membrane-bound \(\alpha\) proteins in the PVN after 7 days of saline or FLX injections indicated as follows: no effect on \(\alpha1\) proteins \([t
value = -1.70, \( p = 0.1109 \), no effect on \( \text{Gai2} \) proteins [t value = -0.49, \( p = 0.6318 \)], no effect on \( \text{Gai3} \) proteins [t value = 0.25, \( p = 0.8105 \), no effect on \( \text{GaO} \) proteins [t value = -0.39, \( p = 0.7001 \)], and no effect on \( \text{Gaz} \) proteins [t value = -0.34, \( p = 0.7354 \)].

The Student’s t-test for cytosol-associated \( \text{Ga} \) proteins in the PVN after 7 days of saline or FLX injections indicated as follows: no effect on \( \text{Gai1} \) proteins [t value = -0.29, \( p = 0.7796 \)], no effect on \( \text{Gai2} \) proteins [t value = -1.04, \( p = 0.598 \)], no effect on \( \text{Gai3} \) proteins [t value = -0.53, \( p = 0.6069 \)], no effect on \( \text{GaO} \) proteins [t value = 0.36, \( p = 0.7215 \)], and no effect on \( \text{Gaz} \) proteins [t value = 0.47, \( p = 0.6472 \)].

The Student’s t-test for membrane-bound \( \text{Ga} \) proteins in the PVN after 14 days of saline or FLX injections indicated as follows: no effect on \( \text{Gai1} \) proteins [t value = -1.46, \( p = 0.1669 \)], no effect on \( \text{Gai2} \) proteins [t value = 1.11, \( p = 0.8952 \)], no effect on \( \text{Gai3} \) proteins [t value = -0.11, \( p = 0.9157 \)], no effect on \( \text{GaO} \) proteins [t value = -1.00, \( p = 0.3337 \)], and no effect on \( \text{Gaz} \) proteins [t value = 0.08, \( p = 0.939 \)].

The Student’s t-test for cytosol-associated \( \text{Ga} \) proteins in the PVN after 14 days of saline or FLX injections indicated as follows: no effect on \( \text{Gai1} \) proteins [t value = 3.11, \( p = 0.1576 \)], no effect on \( \text{Gai2} \) proteins [t value = 1.22, \( p = 0.8015 \)], no effect on \( \text{Gai3} \) proteins [t value = -0.94, \( p = 0.3642 \)], no effect on \( \text{GaO} \) proteins [t value = -0.01, \( p = 0.994 \)], and no effect on \( \text{Gaz} \) proteins [t value = -0.79, \( p = 0.4729 \)].
C. Effects of chronic fluoxetine treatment of peripubertal rats on 5-HT$_{1A}$ receptor-mediated oxytocin responses. (+)-8-OH-DPAT increased plasma levels of oxytocin around 18 times compared with saline. Chronic fluoxetine treatment for either 7 or 14 days did not alter (+)-8-OH-DPAT (0.20 mg/kg) induced increases in plasma levels of oxytocin in the peripubertal rat (figure 24).

The two-way ANOVA for oxytocin for 7 days of treatment indicated no main effect of FLX [$F_{1,49} = 0.16, p = 0.6889$] but a significant main effect of challenge injection [$F_{1,49} = 475.22, p < 0.0001$]. There was no significant interaction between treatment and challenge injection [$F_{1,49} = 0.14, p = 0.7122$]. A Newman-Keuls’ test indicated that (+)-8-OH-DPAT challenge significantly elevated plasma oxytocin levels over saline-challenged in both chronic saline-treated and FLX-treated groups ($p < 0.01$).

The two-way ANOVA for oxytocin for 14 days of treatment indicated no main effect of FLX [$F_{1,46} = 1.57, p = 0.2167$] but a significant main effect of challenge injection [$F_{1,46} = 839.85, p < 0.0001$]. There was no significant interaction between treatment and challenge injection [$F_{1,46} = 0.92, p = 0.344$]. A Newman-Keuls’ test indicated that (+)-8-OH-DPAT challenge significantly elevated plasma oxytocin levels over saline-challenged in both chronic saline-treated and FLX-treated groups ($p < 0.01$).
D. Effects of chronic fluoxetine treatment of peripubertal rats on 5-HT$_{1A}$ receptor-mediated ACTH responses. (+)8-OH-DPAT increased plasma levels of ACTH around 8 times compared with saline. Chronic fluoxetine treatment increased (+)8-OH-DPAT (0.20 mg/kg)-induced increases in plasma levels of ACTH in the peripubertal rat over its respective saline-treated/(+)8-OH-DPAT-challenged group (15% over 7 day and 23% over 14 day) (figure 25).

The two-way ANOVA for ACTH for 7 days of treatment indicated no main effect of FLX [F$_{1,48} = 3.43$, p = 0.0707] but a significant main effect of challenge injection [F$_{1,48} = 385.02$, p < 0.0001]. There was a significant interaction between treatment and challenge injection [F$_{1,48} = 6.29$, p = 0.0158]. A Newman-Keuls’ test indicated that (+)8-OH-DPAT challenge significantly elevated plasma ACTH levels over saline-challenged in both chronic saline-treated and FLX-treated groups (p < 0.01). Additionally, (+)8-OH-DPAT challenge elicited a significantly higher ACTH response in those rats treated with 7 days of FLX over those treated chronically with saline (p < 0.05).

The two-way ANOVA for ACTH for 14 days of treatment indicated a main effect of FLX [F$_{1,44} = 20.40$, p < 0.0001] and a significant main effect of challenge injection [F$_{1,44} = 1084.84$, p < 0.0001]. There was also a significant interaction between treatment and challenge injection [F$_{1,44} = 25.61$, p < 0.0001]. A Newman-Keuls’ test indicated that (+)8-OH-DPAT challenge significantly elevated plasma ACTH levels over saline-challenged in both chronic saline-treated and FLX-
treated groups (p < 0.01). Again, (+)8-OH-DPAT challenge elicited a significantly higher ACTH response in those rats treated with 14 days of FLX over those treated chronically with saline (p < 0.01).

E. Effects of chronic fluoxetine treatment of peripubertal rats on 5-HT$_{1A}$ receptor-mediated and basal levels of pERK in the PVN. In the rats treated for 7 days with fluoxetine, (+)8-OH-DPAT injection increased levels of pERK in the PVN (↑ 200% above basal). (+)8-OH-DPAT challenge after treatment for 14 days with saline (PD 28-41) increased pERK in the PVN, although this response was not as robust (↑ ~50%). For both 7 day and 14 day chronic treatments, FLX had no significant effect on (+)8-OH-DPAT-induced increases in pERK, although basal levels were increased (↑ ~80%) with 7 day treatment (figure 26).

The two-way ANOVA for levels of phosphorylated ERK in the PVN indicated no significant main effect of 7 days of FLX treatment [F$_{1,23} = 1.75$, p = 0.2011] but a significant main effect of challenge injection [F$_{1,23} = 31.30$, p < 0.0001]. There was no significant interaction between treatment and challenge injection [F$_{1,23} = 1.48$, p = 0.2373]. The Newman-Keuls’ test indicated that the (+)8-OH-DPAT challenge significantly elevated pERK in the PVN over the saline-challenged group in both chronic saline-treated (p < 0.01) and chronic FLX-treated (p < 0.05). Although the basal levels of phosphorylated ERK in the PVN increased by about 79% with FLX treatment, this did not reach statistical significance.
The two-way ANOVA for levels of phosphorylated ERK in the PVN indicated no significant main effect of 14 days of FLX treatment \([F_{1,23} = 0.76, p = 0.3934]\) but a significant main effect of challenge injection \([F_{1,23} = 5.19, p = 0.0338]\). There was no significant interaction between treatment and challenge injection \([F_{1,23} = 0.28, p = 0.6047]\). Although the basal levels of phosphorylated ERK in the PVN decreased by about 31\% with chronic fluoxetine treatment, this did not reach statistical significance, nor did 5-HT\(_{1A}\) receptor-stimulated elevations of pERK in chronic saline-treated (\(\uparrow39\%\)) and chronic FLX-treated (\(\uparrow91\%\)) groups.

**F. Effect of chronic fluoxetine of prepubertal rats on plasma levels of testosterone.** Neither 7 nor 14 days of chronic FLX treatment of prepubertal rats had any effect on the plasma levels of testosterone. All treatment groups had average plasma testosterone levels between 50-75 ng/dl (figure 27).

The two-way ANOVA for plasma testosterone levels indicated no significant main effect of treatment time \([F_{1,90} = 1.12, p = 0.2918]\) and no significant main effect of treatment \([F_{1,90} = 0.04, p = 0.8415]\). There was also no significant interaction between treatment time and treatment \([F_{1,90} = 1.24, p = 0.2679]\).
Figure 13. Rat body weights in prepubertal (PD 28), peripubertal (PD 42), and adult (PD > 70) rats. ** indicates significant difference (p < 0.01) from the prepubertal age group; ## indicates significant difference (p < 0.01) from the peripubertal age group (one-way ANOVA and Newman-Keuls multiple-range test). N = 10 per group.
Figure 14. Plasma testosterone levels in prepubertal (PD 28), peripubertal (PD 42), and adult (PD > 70) rats. *, ** indicates significant difference (p < 0.05 and p < 0.01, respectively) from the prepubertal age group; ## indicates significant difference (p < 0.01) from the peripubertal age group (one-way ANOVA and Newman-Keuls multiple-range test). N = 10 per group.
Figure 15. PVN levels of membrane-associated (A) Gai1, (B) Gai2, (C) Gai3, (D) Gaz, and (E) Gao proteins in prepubertal (PD 28), peripubertal (PD 42), and adult (PD > 70) rats. ** indicates significant difference (p < 0.01) from the prepubertal age group (one-way ANOVA and Newman-Keuls multiple-range test). N = 5-8 per group. (F) Representative western blots of Gai1, Gai2, Gai3/Gao, and Gaz from the PVN of prepubertal, peripubertal, and adult rats.
### Ga proteins in PVN

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Table 2. PVN levels of membrane-associated Gai1, Gai2, Gai3, Gao, and Gaz proteins in prepubertal (PD 28), peripubertal (PD 42), and adult (PD > 70) rats. ** indicates significant difference (p < 0.01) from the prepubertal (PD 28) age group (one-way ANOVA and Newman-Keuls multiple-range test).

### Ga proteins in Hippocampus

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Table 3. Hippocampal levels of membrane-associated Gai1, Gai2, Gai3, Gao, and Gaz proteins in prepubertal (PD 28), peripubertal (PD 42), and adult (PD > 70) rats. *, ** indicates significant difference (p < 0.05 and p < 0.01, respectively) from the prepubertal (PD 28) age group; ## indicates significant difference (p < 0.01) from the peripubertal age group (PD 42) (one-way ANOVA and Newman-Keuls multiple-range test).

### Ga proteins in Frontal Cortex

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Table 4. Frontal cortex levels of membrane-associated Gai1, Gai2, Gai3, Gao, and Gaz proteins in prepubertal (PD 28), peripubertal (PD 42), and adult (PD > 70) rats. ** indicates significant difference (p < 0.01) from the prepubertal (PD 28) age group; ## indicates significant difference (p < 0.01) from the peripubertal age group (PD 42) (one-way ANOVA and Newman-Keuls multiple-range test).
Figure 16. (A) The effects of intra-PVN injection with either vehicle or pertussis toxin (PTX) on hypothalamic levels of Gαi1, Gαi2, Gαi3, Gαo, and Gαz proteins. ** indicates significant difference (p < 0.01) from the respective vehicle-treated group (Student’s t-test). N = 9-12 per group. (B) Representative western blots of Gαi1, Gαi2, Gαi3/Gαo, and Gαz from the hypothalamus of peripubertal rats 72 hours after intra-PVN injection of vehicle or PTX.
Figure 17. The effects of intra-PVN injection with either vehicle or pertussis toxin (PTX) on basal and 5-HT$_{1A}$ receptor-mediated increases in plasma oxytocin. ** indicates significant difference (p < 0.01) from the respective saline-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 10-23 per group.
Figure 18. The effects of intra-PVN injection with either vehicle or pertussis toxin (PTX) on basal and 5-HT$_{1A}$ receptor-mediated increases in plasma ACTH. ** indicates significant difference (p < 0.01) from the respective saline-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 11-23 per group.
Figure 19. (A) The effects of intra-PVN injection with either vehicle or pertussis toxin (PTX) on basal and 5-HT_{1A} receptor-mediated increases in pERK in the PVN (two-way ANOVA and Newman-Keuls multiple-range test). N = 5-9 per group. (B) Representative western blot of pERK (above) and ERK (below) from the PVN of peripubertal rats given an intra-PVN injection of either vehicle or PTX and, 72 hours later, given a challenge injection of either saline or (+)8-OH-DPAT.
Figure 20. Plasma testosterone levels were not significantly altered by pertussis toxin (PTX) treatment or acute injection of (+)8-OH-DPAT (two-way ANOVA). N = 8-10 per group.
Figure 21. The effects of (A) 7 day and (B) 14 day treatment with either saline or fluoxetine on rat body weight. *, ** indicates significant difference (p < 0.05 and p < 0.01, respectively) from the saline-treated group (repeated measures ANOVA and Newman-Keuls multiple-range test). N = 24-27 per group.
Figure 22. The effects of 7 day treatment with either saline or fluoxetine on (A) membrane-associated and (B) cytosol-associated levels of Gαi1, Gαi2, Gαi3, Gαo, and Gαz proteins (Student’s t-test). N = 6-8 per group. (C) Representative western blots of membrane-associated (left) and cytosol-associated (right) Gαi1, Gαi2, Gαi3/Gαo, and Gαz from the PVN of peripubertal rats treated with either saline or FLX for 7 days.
Figure 23. The effects of 14 day treatment with either saline or fluoxetine on (A) membrane-associated and (B) cytosol-associated levels of G\(\alpha_i1\), G\(\alpha_i2\), G\(\alpha_i3\), G\(\alpha_o\), and G\(\alpha_z\) proteins (Student’s t-test). N = 7-8 per group. (C) Representative western blots of membrane-associated (left) and cytosol-associated (right) G\(\alpha_i1\), G\(\alpha_i2\), G\(\alpha_i3\)/G\(\alpha_o\), and G\(\alpha_z\) from the PVN of peripubertal rats treated with either saline or FLX for 14 days.
Figure 24. The effects of (A) 7 day and (B) 14 day treatment with saline or fluoxetine on basal and 5-HT₁A receptor-mediated increases in plasma oxytocin. ** indicates significant difference (p < 0.01) from the respective saline-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 8-18 per group.
Figure 25. The effects of (A) 7 day and (B) 14 day treatment with saline or fluoxetine on basal and 5-HT1A receptor-mediated increases in plasma ACTH. ** indicates significant difference (p < 0.01) from the respective saline-challenged group, ## indicates significant difference (p < 0.01) from the saline-treated/(+)8-OH-DPAT-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 8-18 per group.
Figure 26. The effects of (A) 7 day and (B) 14 day treatment with saline or fluoxetine on basal and 5-HT\textsubscript{1A} receptor-mediated increases in levels of pERK in the PVN. *, ** indicates significant difference (p < 0.05 and p < 0.01, respectively) from the respective saline-challenged group). N = 6 per group. (C) Representative western blot of pERK (above) and ERK (below) from the PVN of rats treated with Sal or FLX and subsequently challenged with either saline or (+)8-OH-DPAT. 7D treatment is on the left and 14D treatment is on the right.
Figure 27. Plasma testosterone levels were not significantly altered by treatment with fluoxetine for either 7 or 14 days (two-way ANOVA). N = 22-24 per group.
DISCUSSION

5-HT\textsubscript{1A} receptors on hypothalamic neurons stimulate the release of various hormones and contribute to the serotonergic activation of the hypothalamic-pituitary-adrenal (HPA) axis (Sullivan-Hanley and Van de Kar 2003). 5-HT\textsubscript{1A} receptors in the hypothalamus also activate the mitogen-activated protein kinase (MAPK) ERK pathway (Sullivan et al., 2005; Crane et al., 2007). However, most in vivo studies on 5-HT\textsubscript{1A} receptor signaling in the hypothalamic PVN have focused on adult animal models. In the adult rat PVN, 5-HT\textsubscript{1A} receptors mediate increases in plasma levels of oxytocin and ACTH via pertussis toxin (PTX)-insensitive \(G_\alpha_z\) proteins, whereas 5-HT\textsubscript{1A} receptor-mediated increases in activated ERK (pERK) in the PVN occurs via one or more subtypes of PTX-sensitive \(G_\alpha\) proteins (i.e., \(G_\alpha_{i1,2,3}\) and \(G_\alpha_{o}\) proteins). However, prior to sexual maturation, it is not known if these respective signaling pathways are mediated by the dichotomy of \(G_\alpha\) protein subunits that can be identified as PTX-sensitive vs. PTX-insensitive. Differences in the density or in the regulation of \(G_\alpha\) protein subtypes may underlie any observed developmental differences between peripubertal and adult 5-HT\textsubscript{1A} receptor signaling and receptor desensitization in the PVN. Therefore, in the present studies, we investigated the density, signaling, and regulation of \(G_\alpha\) proteins that have the capacity to couple 5-HT\textsubscript{1A} receptors in the peripubertal rat PVN.
With regards to density, $G\alpha$ protein levels vary in early postnatal development in several brain regions. $G\alpha_1$, $G\alpha_2$, and $G\alpha_3$ proteins reach adult levels around postnatal day 18 in the cortex, thalamus, and hippocampus, whereas $G\alpha_0$ proteins continue to increase past early postnatal development in these regions (Ihnatovych et al., 2002). However, until the present dissertation studies, no studies had examined developmental regulation of $G\alpha_z$ in any brain region, or $G\alpha$ protein levels in the immature hypothalamus. The current data revealed that, consistent with the findings in other brain regions (Ihnatovych et al., 2002), $G\alpha_0$ proteins increased with age. $G\alpha_0$ was present at lower levels in prepubertal (PD 28) and peripubertal (PD 42) PVN, hippocampus, and frontal cortex compared to adult. Also consistent with the literature, levels of $G\alpha_1$, $G\alpha_2$, and $G\alpha_3$ in both prepubertal and peripubertal brain were similar to levels observed in adult brain. We also determined that $G\alpha_z$ levels in the prepubertal and peripubertal PVN, hippocampus, and frontal cortex were comparable to adult. Global changes in $G\alpha_0$, but no other $G\alpha$ protein, may be important with regards to signaling because a relatively lower density of $G\alpha_0$ proteins may lead to a decrease in $G\alpha_0$ coupling to the 5-HT$_{1A}$ receptor in the hypothalamus and other brain regions of younger aged rats as compared to that previously established in the adult rat brain (Mannoury la Cour et al., 2006). Therefore, $G\alpha_0$ proteins may be prevented from performing its specific signaling function. However, multiple $G\alpha$ proteins may subserve the same function in 5-HT$_{1A}$
receptor-mediated signaling pathways in the peripubertal brain (i.e., redundancy in $G\alpha$ mediated pathways); therefore, a lower level of $G\alpha_o$ proteins in younger developmental groups may not necessarily alter $5\text{-HT}_{1A}$ receptor signaling if other $G\alpha$ protein subtypes compensate for the lack of $G\alpha_o$ proteins. The limitation of the western blotting technique used in this experiment is that relative levels of $G\alpha$ proteins cannot be compared to one another. Comparisons can only be made of the levels of a single $G\alpha$ protein at different ages. Another possibility for developmental increases in $G\alpha_o$ is that the other $G\alpha$ proteins ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_z$) may be able to perform a wider array of functions in the pre-adult brain, and the “pruning” of functions as the animal ages only then necessitates an increase in $G\alpha_o$. It also should be noted that the cytosol-associated proteins were not examined in this study. The increase in membrane-associated $G\alpha_o$ protein levels may be due to a redistribution of $G\alpha_o$ proteins from cytosol to membrane as the animal ages rather than a total increase in $G\alpha_o$. However, if $G\alpha_o$ proteins are redistributed from cytosol to membrane in the respective brain regions after sexual maturation, then it would corroborate the evidence indicating the unique regulation of $G\alpha_o$ proteins in the developing brain.

In addition to developmental regulation of $G\alpha$ protein levels, the present studies also indicated that $G\alpha$ proteins exhibit unique responses to drug
treatments. Using a PTX treatment paradigm that had previously been shown to be effective in adult rats, we investigated, in the peripubertal PVN, the role of PTX-sensitive Ga\textsubscript{i/o} proteins in 5-HT\textsubscript{1A} receptor signaling of neuroendocrine responses and phosphorylation of ERK. In adult rats, intra-PVN injection of PTX reduced Ga\textsubscript{i1}, Ga\textsubscript{i2}, Ga\textsubscript{i3}, and Ga\textsubscript{o}, but not Ga\textsubscript{z} in the hypothalamus and inhibited 5-HT1A receptor-mediated phosphorylation of ERK in the PVN (Garcia et al., 2006). Therefore, PTX had been shown to be a useful tool to differentiate signaling by these respective subsets of G proteins (i.e., PTX-sensitive or insensitive). The current data indicate that, in peripubertal rats, an intra-PVN injection of PTX did not reduce all subsets of Ga\textsubscript{i/o} proteins in the hypothalamus (as in the adult) but only reduced Ga\textsubscript{i1} and Ga\textsubscript{i2}, increased Ga\textsubscript{o} protein levels, and did not change Ga\textsubscript{i3} and Ga\textsubscript{z} protein levels in the hypothalamus. Therefore, this study revealed that Ga proteins in the peripubertal hypothalamus may be subject to unique regulation by PTX that prevents reductions in Ga\textsubscript{i3} and increases Ga\textsubscript{o}. However, although Ga protein levels were measured at the level of the hypothalamus and not the PVN, there may have been changes in specific Ga proteins at the level at the PVN that were masked by changes in the whole hypothalamus. However, considering that there were no significant reductions in 5-HT\textsubscript{1A} receptor-mediated neuroendocrine responses, these data suggest that Ga\textsubscript{i3}, Ga\textsubscript{o}, and/or Ga\textsubscript{z} could potentially mediate 5-HT\textsubscript{1A} receptor stimulation of hormone responses in the
PVN of peripubertal rats. We cannot rule out Gαi3 and Gαo proteins in mediating the 5-HT\textsubscript{1A} receptor activation of ERK considering that there was no clear effect of PTX on (+)8-OH-DPAT-induced increases in pERK in the PVN, due to the elevation in basal levels of pERK. However, PTX-induced increases in Gαo may be responsible for the elevations observed in basal levels of activated ERK in the PVN. A difference in the Gαi3/Gαo protein structure or binding ability could be preventing ADP-ribosylation in the peripubertal rat brain. In peripubertal prostate tissue, the ability of PTX to ADP-ribosylate Gαi/o proteins was reduced compared to adults, which may be attributed to reduced binding ability of the G proteins to guanosine triphosphate (GTP) in the peripubertal rats (Chen et al., 1995). Another possibility is that there is a difference in post-translational modifications of the proteins. Fatty acylation of Gαo has been observed in the cortex of postnatal rats (Li et al., 1995); however, a search of the literature suggests there has not been much other research on Gα protein post-translational modifications in the developing brain. Another possibility for the differences observed in the peripubertal hypothalamus with PTX treatment is that a 72-hour incubation time is not sufficient (or is perhaps too short) to functionally inactivate Gαi3 and Gαo proteins. It could be that these proteins are more resilient to ADP-ribosylation and begin to reverse the effect of the toxin or are replenished by transcription and translation of their respective genes before the end of the 3-day time period. Similarly, Gαi3 and Gαo may be compensating
for the loss of Ga1 and Ga2 proteins. Compensatory increases in levels of Ga0 have been observed with knockdown of Ga1-3 proteins in vitro (Krumins and Gilman, 2006). There may also be developmental differences in the effects of PTX.

In the literature, there has been only one other study comparing the effects of PTX in peripubertal vs. adult brain: using adult rat brain membranes in vitro, GABA B receptor/G protein binding was reduced upon incubation with PTX in cortex, hippocampus, and cerebellum but not the striatum. However, when PTX was incubated with peripubertal rat brain membranes, GABA B receptor/G protein binding was reduced in striatum and hippocampus but not cortex or cerebellum (Knott et al., 1993). In sum, Ga proteins (namely Ga3 and Ga0) may not have functionally matured in the peripubertal brain and may possibly impact the signaling of the 5-HT1A receptor in the PVN and other regions in the brain.

To determine the specific Ga protein contribution to 5-HT1A receptor-mediated signaling pathways in the peripubertal PVN, a more selective method is necessary, such as adenoviral-delivered siRNA for Ga3 and Ga0 proteins.

In the pertussis toxin experiment, we also observed a trend towards lower plasma levels of testosterone in all treatment groups, which approximated prepubertal levels as determined in experiment 1. This may be due to additional stress effects of surgery and recovery that all rats from the experiment experienced. Increased activity of the HPA axis via intracerebroventricular injections of CRH or a CRH receptor agonist was shown to delay the onset of
puberty in female rats (Kinsey-Jones et al., 2010). If CRH signaling also plays a role in pubertal timing in male rats, then additional stress may account for this slight reduction in testosterone levels in the cannulated rats.

In a previous study conducted in peripubertal rats, chronic fluoxetine (FLX) injections (once per day for 14 days) reduced 5-HT$_{1A}$ receptor-mediated neuroendocrine responses and selectively reduced G$_{ai3}$ and G$_{ao}$ proteins (Chen et al., 2006); however, any effects of FLX-induced reductions of G$_{ai3}$ and G$_{ao}$ on 5-HT$_{1A}$ receptor-mediated ERK activation were not examined. Therefore, peripubertal rats were given chronic fluoxetine (7 and 14 days) to determine the role of these proteins in 5-HT$_{1A}$ receptor-mediated phosphorylation of ERK in the PVN. Since FLX-induced desensitization of 5-HT$_{1A}$ receptor-mediated neuroendocrine responses had already been established in peripubertal rats, the dose of the challenge drug, (+)8-OH-DPAT, was set at 0.20 mg/kg. This dose is submaximal for the 5-HT$_{1A}$ receptor-mediated phosphorylation of ERK but near the maximum dose necessary for 5-HT$_{1A}$ receptor-mediated neuroendocrine responses (Chapter 3). Therefore, we did not expect to observe a desensitization of either oxytocin or ACTH hormone responses. Although neither 7 nor 14 days of FLX had any effect on (+)8-OH-DPAT (0.20 mg/kg)-induced increases in plasma levels of oxytocin, this combination of either 7 or 14 days FLX treatment with 0.20 mg/kg (+)8-OH-DPAT challenge may have elicited a supersensitive plasma ACTH response. Since this supersensitivity was not observed in the
plasma oxytocin responses, FLX may be inducing changes specific to the CRH receptor system, downstream of 5-HT1A receptor activation on CRH-containing cells in the PVN. FLX treatment has shown to increase CRH mRNA in the PVN (Jorgensen et al., 2002), which could potentially lead to increased release of CRH from the PVN with a maximal dose of the 5-HT1A receptor agonist (+)8-OH-DPAT. With regards to the effects of chronic FLX on ERK activation, neither 7 nor 14 days of FLX had any significant effect on either basal or 5-HT1A receptor-mediated phosphorylation of ERK in the PVN. However, in contrast to the preliminary study that indicated that chronic fluoxetine reduces Gαi3 and Gαo in peripubertal hypothalamus (Chen et al., 2006), the current study revealed that there were no significant changes in any Gα protein subtype (cytosol-associated or membrane-associated) in the PVN with chronic FLX treatment. However, we did replicate the finding that Gαz proteins were not reduced with chronic fluoxetine in the peripubertal hypothalamus. Considering that, in adults, Gαz is the exclusive Gα protein mediator of 5-HT1A receptor-mediated neuroendocrine responses and the only Gα protein that is decreased in the PVN upon chronic FLX treatment, the results from this study indicate very strongly that, in the peripubertal hypothalamus, either Gαz is not the exclusive mediator of 5-HT1A receptor-mediated neuroendocrine responses or FLX-induced desensitization of neuroendocrine responses occur via a different mechanism than that operative in the adult PVN. However, there is also the possibility that the activity, but not
protein level, of Gαz proteins are being reduced with chronic FLX treatment. Phosphorylation of Gαz, which has been observed in other systems (Carlson et al., 1989; Lounsbury et al., 1991), could potentially block its association with Gβγ proteins (Fields and Casey 1995) and subsequent activation by 5-HT₁A proteins. If chronic FLX did induce a phosphorylation of Gαz rather than a reduction in protein levels (as observed in the adult hypothalamus), then Gαz proteins could still play a role in 5-HT₁A receptor-mediated responses in the peripubertal PVN.

Chronic FLX attenuates weight gain in both adult and peripubertal rats. The findings from the current study are in agreement with the current literature that suggests that peripubertal rats are more resilient to the attenuation in weight gain that is prominent in adult rats treated with chronic fluoxetine (Iniguez et al., 2010; Homberg et al., 2011), since the average body weight of peripubertal rats treated for 14 days saline vs. FLX differed by only about 2%. Published studies in adult rats indicate that the average body weight of rats treated for 14 days of saline vs. FLX differ between 5-10% (Raap et al., 1999; Homberg et al., 2010).

Not only do 5-HT₁A receptors signal to phosphorylate the protein kinase ERK in the PVN via a different time-course than that previously established in adults (see Chapter 3 and Crane et al., 2007), but we also have established that the Gα proteins associated with this receptor are subject to both developmental and drug-induced regulation that differ from mechanisms previously established in the adult PVN. These findings have far-reaching implications for the global
signaling of the 5-HT$_{1A}$ receptor in the peripubertal PVN and also for the receptor desensitization and regulation induced by antidepressants and other serotonergic modulators.
CHAPTER 5

IMMUNOHISTOCHEMICAL DETECTION OF OXYTOCIN AND SEROTONIN 1A RECEPTOR-MEDIATED INCREASES IN PHOSPHORYLATED EXTRACELLULAR SIGNAL-REGULATED KINASE ARE LOCALIZED TO DIFFERENT REGIONS OF THE PERIPUBERTAL HYPOTHALAMIC PARAVENTRICULAR NUCLEUS.

ABSTRACT

Serotonin 1A (5-HT_{1A}) receptor activation in the hypothalamic paraventricular nucleus (PVN) stimulates increases in plasma levels of oxytocin and adrenocorticotropic hormone (ACTH) and also increases levels of activated extracellular signal-regulated kinase (ERK). In adult rat PVN, 5-HT_{1A} receptors are localized on oxytocin-containing and CRH-containing cells (Zhang et al., 2004); however, the specific cellular localization of 5-HT_{1A} receptor-mediated activation of ERK in these neuroendocrine cells has not been determined.

Blockade of mitogen activated protein kinase (MAPK) kinase (the upstream activating kinase for ERK) abolishes SSRI-induced desensitization of 5-HT_{1A} receptor-mediated increases of plasma oxytocin responses (Jia et al., 2007). Therefore, it is possible that 5-HT_{1A} receptors can activate ERK in oxytocin-containing cells in the PVN. However, other studies indicating low levels of ERK phosphorylation in oxytocin-containing cells in adult brain (Blume et al., 2009; Wang et al., 2006) suggest otherwise. Previous studies in this dissertation project
demonstrated that 5-HT$_{1A}$ receptor activation can also stimulate increases in plasma hormone responses (oxytocin and ACTH) and phosphorylation of ERK in the peripubertal PVN. However, these studies did not determine the cellular localization of receptor activated ERK in the hypothalamus of peripubertal rats. Therefore, the objective of the current study was to determine, at the cellular level, if 5-HT$_{1A}$ receptor-mediated pERK responses can be detected in oxytocin-containing cells in the peripubertal hypothalamic PVN. In the present study, peripubertal (PD 42) rats received an injection of saline or the 5-HT$_{1A}$ receptor agonist (+)8-OH-DPAT (0.20 mg/kg) 10 minutes prior to sacrifice. Injection with (+)8-OH-DPAT produced marked increases in pERK immunoreactivity in the PVN in peripubertal PVN; however, we did not find any evidence that pERK-positive cells were also immunoreactive for oxytocin. Preliminary data presented here suggest that, in the adult brain, oxytocin and 5-HT$_{1A}$ receptor-mediated pERK were also localized to distinct regions of the PVN. These findings are consistent with previously published data that suggest that pERK stimulation is minimal in oxytocin-containing cells.

In the adult rat hypothalamic PVN, an intact ERK signaling pathway is necessary for SSRI-induced desensitization of 5-HT$_{1A}$ receptor-mediated oxytocin responses. The data from the present studies suggest that SSRI-induced desensitization of 5-HT$_{1A}$ receptor-mediated oxytocin responses occur via ERK activation on non-oxytocin-containing cells and involve transynaptic vs.
intracellular mechanisms. This indicates that 5-HT$_{1A}$ receptor-mediated ERK signaling may play a more widespread role in PVN control, since it could potentially affect the function of cells outside of the small population of cells where ERK is directly activated.

**INTRODUCTION**

The paraventricular nucleus (PVN) is one of several cell groups of the medial hypothalamus and is a center for complex neuroendocrine and autonomic control of the CNS and periphery (Swanson and Sawchenko 1980; Sawchenko and Swanson 1983). The PVN consists of both magnocellular and parvicellular divisions (Swanson and Kuypers 1980). The magnocellular region contains many oxytocin cells, whereas the parvicellular region (medial portion) includes CRH-containing cells as well as some oxytocin-containing cells. Although there are distinct populations of oxytocin-containing and CRH-containing cells within the PVN, oxytocin and CRH do moderately colocalize in a discrete anterior part of the magnocellular division of the nucleus (Sawchenko et al., 1984).

In adult rat PVN, 5-HT$_{1A}$ receptors localize to both oxytocin-containing and CRH-containing cell types (Zhang et al., 2004). 5-HT$_{1A}$ receptor activation increases levels of phosphorylated ERK at the level of the whole PVN in the adult rat (Crane et al., 2007; Sullivan et al., 2005) and peripubertal rat (Chapter 3); however, the cellular localization of 5-HT$_{1A}$ receptor-mediated ERK activation in the hypothalamic PVN has not been determined in any age group. In CRH-
containing cells in the adult rat PVN, increases in pERK have been observed upon stimulation with (1) norepinephrine (via activation of α1 receptors; Khan et al., 2007) and (2) prolactin (Blume et al., 2009). Since blockade of MAPK kinase (the activating kinase for ERK) abolishes SSRI-induced desensitization of 5-HT_{1A} receptor-mediated increases in ACTH responses (Jia et al., 2007), increases in pERK mediated by 5-HT_{1A} receptor activation are most likely contained in CRH-containing cells. MAPK kinase inhibition also abolishes SSRI-induced desensitization of 5-HT_{1A} receptor-mediated oxytocin responses; therefore, it is also likely that 5-HT_{1A} receptors can activate ERK in oxytocin-containing cells in the PVN. However, a study from the literature reveals that, while around 70% of CRH-containing cells also contained phosphorylated ERK, only about 15% of oxytocin-containing cells contained prolactin-stimulated pERK (Blume et al., 2009). A low level of pERK stimulation in oxytocin-containing cells (around 10%) was also demonstrated upon injection with the cytokine interleukin 1β (IL-1β; Wang et al., 2006). Together, these data suggest that oxytocin-containing cells contain low levels of MAPK activity.

5-HT_{1A} receptor activation results in a rapid and prolonged elevation of phosphorylated ERK at the level of the whole PVN in the peripubertal brain (Chapter 3); however, no studies have investigated if 5-HT_{1A} receptor-activated ERK is localized to specific neuroendocrine cells in the PVN of peripubertal rats. Therefore, the purpose of the current study was to determine, at the cellular level,
if 5-HT$_{1A}$ receptor-mediated increases in pERK can be detected in oxytocin-containing cells in the peripubertal hypothalamic PVN. Specifically, the objective was to determine (1) if (+)-8-OH-DPAT induces a measurable or significant increase in phosphorylated ERK in peripubertal PVN using immunohistochemistry and, if so, (2) if 5-HT$_{1A}$ receptor-mediated phosphorylation of ERK is localized to oxytocin-containing cells in the PVN. We also performed preliminary studies to determine the localization of 5-HT$_{1A}$ receptor-stimulated pERK immunoreactivity in oxytocin-containing cells in the adult PVN. In addition, plasma oxytocin and ACTH concentrations were determined to confirm activation of 5-HT$_{1A}$ receptor-mediated responses. Plasma levels of testosterone were also determined to ensure that the stage of sexual maturation were not different between different challenge injection groups.

**MATERIALS AND EXPERIMENTAL PROCEDURES** *(for detailed methods, see Chapter 7)*

**Animals.** Peripubertal (100-125 g; postnatal day 35) and adult (275-300 g; postnatal day 63) male Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN). The determination of post-natal day zero (day of birth) was carried out by the supplier.

**Drugs.** (+)-8-Hydroxy-2-(di-n-propylamino)tetralin hydrobromide [(+)-8-OH-DPAT] was purchased from Tocris Cookson Inc. (Ellisville, MO) and was
dissolved in saline (0.9% NaCl). (+)8-OH-DPAT was injected at a volume of 1 ml/kg and was freshly prepared immediately prior to the injections.

**Experiment 1: Localization of 5-HT$_{1A}$ receptor-mediated activation of ERK in the peripubertal and adult PVN.** Peripubertal and adult animals were assigned randomly to treatment groups and were handled for at least 3 days before injections to minimize stress. On postnatal day (PD) 42 for peripubertal animals and PD 70 for adult animals, the rats were given an injection of saline (1 ml/kg, s.c.) or (+)8-OH-DPAT (0.20 mg/kg, s.c.) with sacrifice occurring 10 minutes after injection for peripubertal rats (the optimal sacrifice time for observing elevations in pERK in peripubertal PVN with western blotting, see Chapter 3) or 5 minutes after injection for adult animals (Crane et al., 2007). As an additional control, two peripubertal animals were also handled for 3 days but were not injected. The trunk blood was collected in tubes and prepared for subsequent determination of plasma levels of oxytocin, ACTH, and testosterone. Brains were harvested within one minute of the decapitation and were preserved using the immersion fixation procedure (adapted from Khan and Watts, 2004).

**RESULTS**

1. **Experiment 1: Localization of 5-HT$_{1A}$ receptor-mediated activation of ERK in the PVN.** The objectives of this experiment were to determine (1) if (+)8-OH-DPAT induces phosphorylation of ERK in the PVN of peripubertal rats using immunofluorescence and, if so, (2) if 5-HT$_{1A}$ receptor-mediated phosphorylation
of ERK is localized to oxytocin-containing cells in the PVN. We also performed preliminary studies to determine the localization of 5-HT\textsubscript{1A} receptor-stimulated pERK immunoreactivity in oxytocin-containing cells in the adult PVN.

A. Immunoreactivity for 5-HT\textsubscript{1A} receptor-mediated phosphorylation of ERK did not localize to oxytocin-immunoreactive cells in the peripubertal hypothalamic PVN. As shown in figure 28, injection with (+)8-OH-DPAT increased pERK immunoreactivity in the peripubertal PVN; however, none of these cells were also immunoreactive for oxytocin. This finding was also confirmed with higher powered-magnification using confocal imaging (figure 29). The oxytocin-containing cells concentrate more laterally in magnocellular divisions of the PVN, while the pERK-containing cells were concentrated medially (closer to the third ventricle) in parvocellular cell groups.

B. The effect of (+)8-OH-DPAT on the measure of integrated density for pERK and oxytocin labeling in the peripubertal PVN. As shown in table 5, (+)8-OH-DPAT had no effect on the integrated density (defined as the product of the pixel area and the optical density) in the peripubertal PVN. However, due to the intense baseline signal of fibers and vessels with the pERK antibody in the PVN (Khan et al., 2007), evaluating the integrated density alone for the whole PVN in peripubertal rats may not be as sensitive as counting labeled cells (as shown in section C below).
The Student’s t-test for oxytocin indicated no significant effect of (+)8-OH-DPAT challenge compared to saline challenge [t value = 0.84, p = 0.4204]. The Student’s t-test for pERK also indicated no significant effect of (+)8-OH-DPAT challenge compared to saline challenge [t value = -0.07, p = 0.9474].

C. The effect of (+)8-OH-DPAT on number of cells labeled for pERK and oxytocin in the PVN of peripubertal rats. As shown in table 6, particle analysis using ImageJ software (identifying round cells and eliminating narrow fiber or vessel labeling) indicated that (+)8-OH-DPAT had no effect on the number of oxytocin-immunoreactive cells, but increased the number of pERK-immunoreactive cells ~2.9 fold above saline-injected controls in the peripubertal PVN.

The Student’s t-test for oxytocin indicated no significant effect of (+)8-OH-DPAT injection compared to saline injection [t value = 1.10, p = 0.2975]. The Student’s t-test for pERK, however, indicated a significant effect of (+)8-OH-DPAT challenge compared to saline challenge [t value = -3.19, p = 0.0129]. Therefore, these data suggest that stimulation of 5-HT$_{1A}$ receptors in vivo induces a measurable increase in the number of pERK immunoreactive cells but has no effect on the number of cells that were immunoreactive for oxytocin in the peripubertal PVN.

D. Immunoreactivity for 5-HT$_{1A}$ receptor-mediated phosphorylation of ERK was not found in oxytocin-containing cells in the adult hypothalamic PVN.
Preliminary data suggest that, in the adult PVN, injection with 0.20 mg/kg (+)8-OH-DPAT markedly increased pERK-immunoreactivity. However, none of these cells were also immunoreactive for oxytocin (figure 30).

E. (+)8-OH-DPAT increased plasma levels of oxytocin in peripubertal and adult rats. Plasma hormone levels were measured in order to confirm that the in vivo injection with 0.20 mg/kg (+)8-OH-DPAT stimulated 5-HT1A receptors in the PVN. As shown in figure 31, challenge with (+)8-OH-DPAT increased plasma levels of oxytocin in both peripubertal (~36 fold over saline) and adult (~13 fold over saline) rats.

F. (+)8-OH-DPAT increased plasma levels of ACTH in peripubertal and adult rats. As shown in figure 32, injection of 0.20 mg/kg (+)8-OH-DPAT increased plasma levels of ACTH in both peripubertal (~8 fold over saline) and adult (~4 fold over saline) rat groups. These data indicate that the in vivo injection of 0.20 mg/kg (+)8-OH-DPAT stimulated neuroendocrine responses in both peripubertal and adult rats, presumably via 5-HT1A receptor-mediated activation in the PVN.

G. (+)8-OH-DPAT had no effect on plasma levels of testosterone in peripubertal and adult rats. Plasma levels of testosterone were measured in order to ensure that the state of sexual maturation was consistent within treatment groups and age groups. (+)8-OH-DPAT had no effect on the plasma levels of testosterone in peripubertal or adult animals compared to saline-challenged rats or untreated
routers. As expected, adult rats had higher plasma levels of testosterone than did peripubertal rats (figure 33).
Figure 28. Representative images of oxytocin, pERK, and overlays in peripubertal PVN of rats injected with either (A) saline or (B) 0.20 mg/kg (+)8-OH-DPAT (untreated not shown). Magnification = 10x; yellow scale bar = 100 μm.
**Peripubertal PVN**

**Figure 29.** Representative confocal images of oxytocin-immunoreactive cells, pERK immunoreactive cells, and overlays of immunoreactive cells taken from three different randomly chosen areas of the PVN from one (+)8-OH-DPAT-injected (0.20 mg/kg) peripuberal rat. Red = oxytocin-immunoreactive cells; green = pERK-immunoreactive cells.
Effects of 5-HT$_{1A}$ Receptor Stimulation on Integrated Density of Immunoreactivity in Peripubertal PVN

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oxytocin</th>
<th>pERK</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>4.5</td>
<td>1.1</td>
<td>2</td>
</tr>
<tr>
<td>Saline</td>
<td>3.8 ± 0.3</td>
<td>3.4 ± 1.2</td>
<td>6</td>
</tr>
<tr>
<td>(+)8-OH-DPAT</td>
<td>3.3 ± 0.6</td>
<td>3.5 ± 0.9</td>
<td>6</td>
</tr>
</tbody>
</table>

**Table 5.** Average integrated density (x100) of oxytocin immunoreactivity and 5-HT$_{1A}$ receptor-stimulated pERK immunoreactivity in the peripubertal PVN. Numbers are displayed as the average integrated density (optical density multiplied by the area of a 850x950 pixel section) of each group ± SEM for groups with more than 2 rats. Student t-test indicated no effect of (+)8-OH-DPAT challenge injection for either oxytocin or pERK integrated density of immunoreactivity compared to rats injected with saline.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oxytocin</th>
<th>pERK</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>124.7</td>
<td>9.3</td>
<td>2</td>
</tr>
<tr>
<td>Saline</td>
<td>115.7 ± 8.9</td>
<td>15.1 ± 5.9</td>
<td>5</td>
</tr>
<tr>
<td>(+)8-OH-DPAT</td>
<td>96.2 ± 17.5</td>
<td>44.0 ± 6.9 *</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 6. Average cell density of 850x950 pixel area for oxytocin-immunoreactive cells and cells immunoreactive for 5-HT1A receptor-stimulated pERK in the PVN of peripubertal rats. Numbers are displayed as the average cell number of each group ± SEM for groups with more than 2 rats. * p < 0.05 with respect to saline (Student’s t-test).
Figure 30. Representative images of oxytocin, pERK, and overlays in adult PVN from animals that have been injected with (A) saline or (B) (+)8-OH-DPAT (0.20 mg/kg). Magnification = 10x; white scale bar = 100 μm.
Figure 31. Plasma oxytocin responses in (A) peripubertal and (B) adult rats used in immunohistochemistry experiments.
Figure 32. Plasma ACTH responses in (A) peripubertal and (B) adult rats used in immunohistochemistry experiments.
**Figure 33.** Plasma levels of testosterone in peripubertal and adult rats used in immunohistochemistry experiments.
DISCUSSION

Activation of 5-HT$_{1A}$ receptors via *in vivo* injection with (+)8-OH-DPAT increases plasma levels of oxytocin and ACTH and increases levels of pERK in the PVN (as measured by western blotting) in both peripubertal and adult brains (Chapter 3 and Crane et al., 2007). Although increases in pERK mediated by neuroactive ligands such as norepinephrine or prolactin have been identified in specific neuroendocrine-containing cell types in the PVN, 5-HT$_{1A}$ receptor-mediated activation of ERK has not been demonstrated in the specific cell types in the PVN of rats at any age. The study described in this chapter is the first to demonstrate 5-HT$_{1A}$ receptor-mediated increases in pERK immunoreactivity in the peripubertal PVN using immunohistochemistry. The present study determined that 5-HT$_{1A}$ receptor-mediated increases in pERK immunoreactivity did not localize to cells that were immunoreactive for oxytocin. Preliminary data obtained in this study also suggest that 5-HT$_{1A}$ receptor-induced increases in pERK immunoreactivity and oxytocin immunoreactivity were contained in distinct cell groups in the adult PVN, indicating this finding is not unique to the immature brain.

The current preliminary data are consistent with the literature in that receptor-activated ERK localizes to very few oxytocin-containing cells (Wang et al., 2006; Blume et al., 2009). Since 5-HT$_{1A}$ receptor-mediated increases in pERK immunoreactivity and oxytocin immunoreactivity were localized to distinct cell
groups in the peripubertal PVN, 5-HT\textsubscript{1A} receptor-mediated pERK and 5-HT\textsubscript{1A} receptor-mediated increases in plasma oxytocin responses are likely mediated by separate pathways in different cell populations. Similarly, little to no localization of 5-HT\textsubscript{1A} receptor-activated ERK on oxytocin-containing cells in the adult PVN support previous findings indicating that 5-HT\textsubscript{1A} receptors mediate increases in plasma levels of oxytocin and increases in ERK activation via independent pathways in the adult PVN (Garcia et al., 2006).

In the adult PVN, ERK signaling is necessary for SSRI-induced desensitization of 5-HT\textsubscript{1A} receptor-mediated oxytocin (and ACTH) neuroendocrine responses, since blockade of MAPK kinase (MEK), the activating kinase for ERK, abolishes SSRI-induced desensitization of 5-HT\textsubscript{1A} receptor-mediated hormone responses and associated reductions in G\textsubscript{z} proteins (Jia et al., 2007). The current results imply that activated ERK in the adult PVN may function via an indirect, cell-to-cell mechanism rather than a direct, intracellular mechanism to mediate SSRI-induced desensitization of 5-HT\textsubscript{1A} receptor-mediated oxytocin responses. In the peripubertal PVN, it is unknown if ERK is required for SSRI-induced desensitization of 5-HT\textsubscript{1A} receptor mediated oxytocin responses. If ERK is necessary, then a similar cell-to-cell mechanism for SSRI-induced desensitization of 5-HT\textsubscript{1A} receptor-mediated oxytocin responses may be operant in the immature brain as suggested in the adult brain.
The labeling of CRH in the PVN has proved to be challenging, and many labs have found it necessary to treat rats *in vivo* with colchicine in order to prevent axonal transport of CRH, thereby concentrating the CRH in the cells. It is also standard procedure in most labs to perfuse animals rather than post-fix the brain tissue. These techniques may be necessary to determine the extent of 5-HT$_{1A}$ receptor-mediated pERK on CRH-containing cells in both peripubertal and adult PVN. Despite these issues, we have gathered some preliminary data (not shown) that indicates that 5-HT$_{1A}$ receptor-mediated activation of ERK can occur on CRH-containing cells in the peripubertal PVN. 5-HT$_{1A}$ receptor-mediated increases in pERK immunoreactivity may extensively colocalize with CRH immunoreactivity, since 70% of cells immunopositive for CRH have been shown to express prolactin-induced ERK activation (Blume et al., 2009). If 5-HT$_{1A}$ receptor-mediated pERK extensively localizes to cells that are immunoreactive for CRH, then it could be possible that ERK activation could potentially lead to 5-HT$_{1A}$ receptor-mediated plasma levels of ACTH in the peripubertal age group (i.e., 5-HT$_{1A}$ receptor-mediated pathways are dependent on one another in CRH-containing cells in the peripubertal PVN). Furthermore, if we were to find that ERK is necessary for SSRI-induced desensitization of 5-HT$_{1A}$ receptor-mediated increases in plasma oxytocin and ACTH responses in the peripubertal PVN, CRH cells could have the capacity to send out projections or a local agent to regulate nearby oxytocin-containing cells and their outputs to the pituitary gland.
while using an intracellular mechanism to desensitize CRH-containing cell outputs. Potentially, the small population of cells in the PVN that contain 5-HT$_{1A}$ receptor-mediated pERK could exert a wide range of control over multiple cell subtypes within the PVN and in other areas of the hypothalamus.
CHAPTER 6

INITIAL IDENTIFICATION OF IN VIVO SEROTONIN 1A RECEPTOR ACTIVATION OF PROTEIN KINASE B (AKT) IN THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS OF PERIPUBERTAL RATS.

ABSTRACT

Protein kinase B (Akt) and its downstream signaling effector glycogen synthase kinase 3β (GSK3β) have been associated with cell survival and may represent a common target for antidepressants and other drugs used to treat mood disorders (Beaulieu et al., 2009). Serotonin 1A (5-HT1A) receptor agonists increase levels of phosphorylated Akt (pAkt) in several cell lines (Adayev et al., 1999; Cowen et al., 2005; Hsiung et al., 2005) and in adult hippocampus in vivo (Polter et al., 2010). To date, no preclinical studies have determined 5-HT1A receptor-mediated activation of the Akt signal transduction pathway in the hypothalamus in any age group. Since hypothalamic dysfunction has been observed in studies of childhood and adolescent stress and depression (Vaillancourt et al., 2007; Lopez-Duran et al., 2009; Vigil et al., 2010; Hankin et al., 2010), the present studies investigated 5-HT1A receptor signaling of the Akt signaling pathway in the peripubertal hypothalamic paraventricular nucleus (PVN) with respect to: (1) the onset and longevity of increases in pAkt at two activating residues (Thr308 and Ser473) by two different classes of 5-HT1A...
receptor agonists, (2) potential agonist-dependent differences in the potency and efficacy of different 5-HT_{1A} receptor agonists to phosphorylate Akt at each of the respective phosphorylation sites, (3) the 5-HT_{1A} receptor selectivity of (+)8-OH-DPAT-mediated increases of pAkt, (4) the potential role of pertussis toxin-sensitive Gα protein subtypes in 5-HT_{1A} receptor-mediated increases of pAkt, (5) the effects of chronic (7 and 14 days) fluoxetine (FLX) on 5-HT_{1A} receptor-mediated increases in phosphorylated Akt, and (6) the effects of chronic (7 and 14 days) FLX on 5-HT_{1A} receptor-mediated increases in GSK3β.

Together, these data provide the first in vivo demonstration in the peripubertal PVN that 5-HT_{1A} receptors can activate Akt via phosphorylation at both Thr308 and Ser473. Given the previous studies demonstrating that 5-HT_{1A} receptors can stimulate hormone responses and also activate ERK in the peripubertal PVN (Chapter 3), these findings are the first to show that 5-HT_{1A} receptors can mediate the concomitant activation of three signaling pathways in peripubertal hypothalamic PVN. (+)8-OH-DPAT had full agonist activity and tandospirone only partial agonist activity on the phosphorylation of Akt at both Thr308 and Ser473, despite these both drugs displaying full agonist activity at 5-HT_{1A} receptor-mediated neuroendocrine responses and ERK activation in the peripubertal PVN (Chapter 3). With regards to receptor selectivity, pretreatment with the 5-HT_{1A} receptor antagonist WAY100635 completely blocked (+)8-OH-DPAT-induced elevations of pAkt at Ser473 in the PVN. Intra-PVN injection of
pertussis toxin (which only reduced Gα1 and Gα2 proteins in the peripubertal hypothalamus, see Chapter 4) precluded any significant increase in (+)8-OH-DPAT-induced phosphorylation of Akt (at Ser473) compared to its respective basal level. Therefore, Gα1 and Gα2 may play a role in Akt phosphorylation; however, since pertussis toxin also produced a non-significant 23% increase in basal levels of phosphorylated Akt at Ser473, this could have precluded obtaining a significant (+)8-OH-DPAT-induced increase above basal levels. Lastly, chronic FLX does not alter basal levels of Akt, basal levels of GSK3β, or 5-HT1A receptor-mediated activation of Akt. However, chronic FLX can selectively regulate 5-HT1A receptor-mediated phosphorylation of GSK3β, implicating phosphatases or a unique mechanism in FLX-induced GSK3β desensitization in the peripubertal PVN.

INTRODUCTION

Akt (protein kinase B) is a protein kinase involved in cell survival, apoptosis, protein synthesis, insulin signaling, and glucose transport (Franke et al., 2003). Receptor tyrosine kinases (RTKs) typically initiate the activation of Akt, although stimulation of G protein coupled receptors such as dopamine D2 receptors (Beaulieu et al., 2005) and 5-HT1A receptors (Adayev et al., 1999; Cowen et al., 2005; Hsiung et al., 2005) has recently been shown to activate this pathway \textit{in vitro}. Receptor activation stimulates phosphoinositol 3-kinase (PI3K) to catalyze the formation of phosphatidylinositol(3,4,5)P3 (PIP3). PIP3 binds to and brings
phosphoinositide-dependent kinase 1 (PDK1) and Akt into close proximity at the cell membrane, where PDK1 then phosphorylates Akt at Thr308 (Alessi et al., 1997; Anderson et al., 1998; Stephens et al., 1998). Akt is also phosphorylated in the hydrophobic motif at Ser473 by a complex containing rictor and mTOR proteins (Sarbossov et al., 2005). Although the exact sequence of Akt phosphorylation at Thr308 and Ser473 is still under investigation and is likely to differ with respect to brain region and cell type, both sites must be phosphorylated in order for Akt to be fully activated (Alessi et al., 1996; Andjelkovic et al., 1996). Downstream, activated Akt phosphorylates and inhibits glycogen synthase kinase 3 (GSK3) (Cross et al., 1995), an enzyme that regulates the function of a multitude of proteins, including metabolic, signaling, and structural proteins (Grimes and Jope 2001). GSK3 exists in 2 isoforms, GSK3α and GSK3β, and are both constitutively active until phosphorylation by Akt (Woodgett 1990; Sutherland et al., 1993). Although both isoforms are highly expressed in brain (Woodgett 1990), GSK3β has received particular attention due to the ability of lithium and other drugs used to treat bipolar and other mood disorders to phosphorylate and inactivate GSK3β (Klein and Melton 1996; DeSarno et al 2002; Zhang et al., 2003; Gould et al., 2004; O’Brien et al., 2004; Li et al., 2007; Fatemi et al., 2009).

Dysfunction of the PI3K/Akt/GSK3 pathway has been implicated in the pathology of psychological disorders including chronic stress, depression, and
schizophrenia (Beaulieu et al., 2009). For example, the activity of PI3K and Akt were significantly decreased in the occipital cortex of suicide victims (with no recent psychotropic drug consumption) compared to controls (Hsiung et al., 2003). In addition, decreases were observed in the activity of Akt (with subsequent increased activation of GSK3) in schizophrenic patients (Emamian et al., 2004) and in a mouse model of schizophrenia (Beaulieu et al., 2004). In a rat model of chronic stress, levels of pAkt were increased in the hypothalamus, hippocampus, and striatum, with the largest increases observed in the hypothalamus (Lee et al., 2006), a region containing 5-HT$_{1A}$ receptors that stimulate hormone responses and activate ERK, but have yet to be studied with respect to Akt activation.

5-HT$_{1A}$ receptors can activate the PI3K/Akt/GSK3 pathway in vitro and in vivo. 5-HT$_{1A}$ receptors increase the phosphorylation of Akt in both neuronal (Adayev et al., 1999; Cowen et al., 2005) and non-neuronal (Hsiung et al., 2005) cell cultures. The 5-HT$_{1A}$ receptor agonist 8-OH-DPAT has recently been shown to increase the levels of phosphorylated Akt and GSK3β in vivo in the adult hippocampus, although the specificity of 5-HT$_{1A}$ receptors for this response has yet to be determined (Polter et al., 2010 and personal communications). Acute SSRI treatment increases levels of phosphorylated GSK3β in mouse frontal cortex, hippocampus, and striatum, which is thought to be due to increased levels of 5-HT stimulating post-synaptic 5-HT$_{1A}$ receptors to mediate subsequent
activation of Akt (Li et al., 2004). These data indicate that 5-HT\textsubscript{1A} receptors \textit{in vivo} likely activate the Akt signaling pathway in the brain and that this signaling cascade may be involved with the effects of antidepressants.

5-HT\textsubscript{1A} receptor-mediated phosphorylation of Akt and its downstream target GSK3\textbeta{} has not been demonstrated in the hypothalamus in an \textit{in vivo} model. Given the potential role of Akt in stress responses and effects of antidepressants, the present studies investigated 5-HT\textsubscript{1A} receptor signaling of the Akt signaling pathway in the peripubertal PVN with respect to: (1) the onset and longevity of 5-HT\textsubscript{1A} receptor-induced phosphorylation of Akt at Thr308 and Ser473 by two classes of 5-HT\textsubscript{1A} receptor agonists, (2) potential agonist-dependent differences in the potency and efficacy of different 5-HT\textsubscript{1A} receptor agonists to phosphorylated Akt at each of its respective phosphorylation sites, (3) the 5-HT\textsubscript{1A} receptor selectivity of (+)8-OH-DPAT-mediated increases in pAkt, (4) the potential role of pertussis toxin-sensitive G\alpha{} protein subtypes in 5-HT\textsubscript{1A} receptor-mediated increases in pAkt, (5) the effects of chronic (7 and 14 days) FLX on 5-HT\textsubscript{1A} receptor-mediated phosphorylation of Akt, and (6) the effects of chronic (7 and 14 days) FLX on 5-HT\textsubscript{1A} receptor-mediated phosphorylation of GSK3\textbeta{.}
MATERIALS AND EXPERIMENTAL PROCEDURES (for detailed methods, see Chapter 7)

Animals. Peripubertal male Sprague-Dawley rats (100-125 g) were purchased from Harlan (Indianapolis, IN). The determination of post-natal day zero (day of birth) was carried out by the supplier.

Drugs. (+)-8-Hydroxy-2-(di-n-propylamino)tetralin hydrobromide [(+)8-OH-DPAT] was purchased from Tocris Cookson Inc. (Ellisville, MO). Tandospirone citrate was a generous gift of Dr. K. Matusubara, Asahikawa Medical College, Japan. (+)8-OH-DPAT and tandospirone were dissolved in saline (0.9% NaCl) and were injected at a volume of 1 ml/kg. For the dose-response experiment, the highest dose of drug was dissolved in saline and lower concentrations were made by serial dilution. N-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide maleate salt (WAY100635) was purchased from Sigma (catalog number W108), and was dissolved in saline (0.9% NaCl) and injected at a volume of 1 ml/kg. Pertussis toxin (PTX; Bordetella pertussis) was purchased from EMD Chemicals, Gibbstown, NJ (catalog number 516560), and was diluted to a concentration of 1 μg/μl with ddH₂O. Fluoxetine hydrochloride was purchased from LKT Laboratories, Inc., St. Paul, MN (catalog number F4780), and was dissolved in saline (0.9% NaCl) to 10 mg/kg and was injected at a volume of 2 ml/kg. All drugs were freshly prepared immediately prior to the injections.
**Experiment 1: Time-dependent 5-HT<sub>1A</sub> receptor-mediated phosphorylation of Akt at Thr308 and Ser473.** At least 3 days of handling preceded any injections. Peripubertal male Sprague-Dawley rats (post-natal day 42) were injected with either saline (1 ml/kg, s.c.), the aminotetralin agonist (+)8-OH-DPAT (0.20 mg/kg, s.c.), or the azapirone agonist tandospirone (10 mg/kg, s.c.). The animals were sacrificed by decapitation at 5, 10, 15, 20, or 30 minutes post-injection. The brains were carefully removed from the skull within one minute of the decapitation and were quickly frozen by submersion in chilled 2-methylbutane (-35°C) for 1 minute and then subsequently kept in dry ice for an additional 10 minutes. Each brain was then securely wrapped and stored at -80°C prior to dissection for Western blot studies for the determination of pAkt/Akt levels in the PVN.

**Experiment 2: Dose-dependent 5-HT<sub>1A</sub> receptor-mediated phosphorylation of Akt at Thr308 and Ser473.** At least 3 days of handling preceded any injections. Peripubertal male Sprague-Dawley rats (post-natal day 42) were injected with either saline (1 ml/kg, s.c.), (+)8-OH-DPAT (0.04, 0.10, 0.20, or 0.50 mg/kg, s.c.), or tandospirone (1, 3, 10, or 20 mg/kg, s.c.) and were sacrificed by decapitation at 10 minutes post-injection. The brains were carefully removed from the skull within one minute of the decapitation and were quickly frozen by submersion in chilled 2-methylbutane (-35°C) for 1 minute and then subsequently kept in dry ice for an additional 10 minutes. Each brain was then securely wrapped and
stored at -80°C prior to dissection for Western blot studies for the determination of pAkt/Akt levels in the PVN.

**Experiment 3: Determination of 5-HT\textsubscript{1A} receptor specificity of (+)8-OH-DPAT-mediated phosphorylation of Akt at Ser473.** At least 3 days of handling preceded any injections. Peripubertal male Sprague-Dawley rats (post-natal day 47) were injected with either saline (1 ml/kg, s.c.) or the 5-HT\textsubscript{1A} receptor antagonist WAY100635 (0.10 mg/kg, s.c.) 30 minutes prior to a challenge injection with either saline (1 ml/kg, s.c.) or (+)8-OH-DPAT (0.20 mg/kg, s.c.). The animals were sacrificed by decapitation at 15 minutes post-injection. The brains were carefully removed from the skull within one minute of the decapitation and were quickly frozen by submersion in chilled 2-methylbutane (-35°C) for 1 minute and then subsequently kept in dry ice for an additional 10 minutes. Each brain was then securely wrapped and stored at -80°C prior to dissection for Western blot studies for the determination of pAkt/Akt levels in the PVN.

**Experiment 4: Determination of the role of PTX-sensitive Gα proteins in 5-HT\textsubscript{1A} receptor-mediated phosphorylation of Akt at Ser473 in the peripubertal PVN.** Cannula implantation and administration of PTX was performed according to procedures previously established in adult rats (Garcia et al., 2006), and detailed in Chapter 7. Seventy-two hours after intracerebral injection of vehicle (100 mM sodium phosphate + 500 mM NaCl, 0.5 μl/side) or PTX (1
μg/μl, 0.5 μl/side), the rats were given a systemic challenge injection of saline (1 ml/kg) or (+)8-OH-DPAT (0.20 mg/kg) and were sacrificed by decapitation 15 minutes later (postnatal day 42). The brains were carefully removed from the skull within one minute of the decapitation and were quickly frozen by submersion in chilled 2-methylbutane (-35°C) for 1 minute and then subsequently kept in dry ice for an additional 10 minutes. Each brain was then securely wrapped and stored at -80°C prior to dissection for Western blot studies for the determination of pAkt/Akt levels in the PVN.

**Experiment 5: Determination of chronic FLX-induced effects on (A) basal and 5-HT\textsubscript{1A} receptor-mediated increases of pAkt (Thr308) and (B) basal and 5-HT\textsubscript{1A} receptor-mediated increases pGSK3β in the peripubertal PVN.** Rats were given an i.p. injection of saline (2 ml/kg) or FLX (10 mg/kg), once per day for either 7 (PD 35-41) or 14 days (PD 28-41). Eighteen hours after the last injection, the rats (postnatal day 42) were given a systemic challenge injection of saline (1 ml/kg) or (+)8-OH-DPAT (0.2 mg/kg) and were sacrificed by decapitation 10 minutes later. The brains were carefully removed from the skull within one minute of the decapitation. The brains were quickly frozen by submersion in chilled isopentane for 1 minute and then subsequently kept in dry ice for an additional 10 minutes. Each brain was then securely wrapped and stored at -80°C prior to dissection for Western blot studies for the determination of pAkt (Thr308)/Akt and pGSK3β/GSK3β levels in the PVN.
RESULTS

1. **Experiment 1: 5-HT\textsubscript{1A} receptor-mediated time-dependent phosphorylation of Akt at Thr 308 and Ser473.** The specific objectives of this experiment were to: (1) determine the onset and longevity of the 5-HT\textsubscript{1A} receptor-mediated increases in (A) pAkt at Thr308 and (B) pAkt at Ser473 in PVN; and (2) identify potential differences in onset and longevity of the different 5-HT\textsubscript{1A} receptor agonists to increase (A) pAkt at Thr308 and (B) pAkt at Ser473 in PVN.

A. **Time-course of (+)8-OH-DPAT-induced increases in pAkt (Thr308) in the peripubertal rat.** As shown in figure 34, a single injection of (+)8-OH-DPAT (0.20 mg/kg) produced rapid (≤ 5 minutes) and prolonged increases in pAkt at Thr308. The magnitude of elevation of (+)8-OH-DPAT-induced pAkt at Thr308 was about 300% over saline baseline.

The two-way ANOVA for (+)8-OH-DPAT-induced increases in pAkt (Thr308) in the PVN indicated a significant main effect of challenge drug (saline or (+)8-OH-DPAT) \([F_{1,78} = 53.74, p < 0.0001]\) but no significant main effect of post-injection time \([F_{4,78} = 1.19, p = 0.3219]\). There was also no significant interaction between challenge drug and post-injection time \([F_{4,78} = 1.19, p = 0.3219]\). A Newman-Keuls’ test indicated that (+)8-OH-DPAT increased pAkt at Thr308 in the PVN over saline at 5, 10, and 15 minutes \(p < 0.01\), and 20 minutes \(p < 0.05\).

B. **Time-course of tandospirone-induced increases in pAkt (Thr308) in the peripubertal rat.** As shown in figure 35, a single injection of tandospirone (10 mg/kg)
mg/kg) produced rapid (≤ 5 minutes) and prolonged increases in pAkt at Thr308. However, the magnitude of elevation of pAkt at Thr308 over saline induced by (+)8-OH-DPAT was much greater (↑300%, figure 34) than that induced by tandospirone (↑85%).

The two-way ANOVA for tandospirone-induced increases in pAkt (Thr308) in the PVN indicated a significant main effect of challenge drug (saline or tandospirone) \( [F_{1,78} = 16.551, p = 0.0001] \) but no significant main effect of post-injection time \( [F_{4,78} = 0.38, p = 0.8200] \). There was also no significant interaction between challenge drug and post-injection time \( [F_{4,78} = 0.38, p = 0.8200] \). A Newman-Keuls’ test indicated that tandospirone increased pAkt at Thr308 in the PVN over saline at 10 minutes (p < 0.05).

C. **Time-course of (+)8-OH-DPAT-induced increases in pAkt (Ser473) in the peripubertal rat.** As shown in figure 36, a single injection of (+)8-OH-DPAT (0.20 mg/kg) produced a rapid (≤ 5 minutes) increase (~↑60% over saline) in pAkt at Ser473 that returned to baseline by 30 minutes post-injection.

The two-way ANOVA for (+)8-OH-DPAT-induced increases in pAkt (Ser473) in the PVN indicated a significant main effect of challenge drug (saline or (+)8-OH-DPAT) \( [F_{1,79} = 27.33, p < 0.0001] \) but no significant main effect of post-injection time \( [F_{4,79} = 1.63, p = 0.1771] \). There was also no significant interaction between challenge drug and post-injection time \( [F_{4,79} = 1.63, p = 0.1771] \). A
Newman-Keuls’ test indicated that (+)8-OH-DPAT increased pAkt at Ser473 in the PVN over saline at 5 and 10 minutes (p < 0.01), and 15 minutes (p < 0.05).

D. **Time-course of tandospirone-induced increases in pAkt (Ser473) in the peripubertal rat.** As shown in figure 37, a single injection of tandospirone (10 mg/kg) produced a rapid (≤ 5 minutes) increase (~↑60% over saline) in pAkt at Ser473 that was comparable in magnitude than that induced by (+)8-OH-DPAT (figure 36).

The two-way ANOVA for tandospirone-induced increases in pAkt (Ser473) in the PVN indicated a significant main effect of challenge drug (saline or tandospirone) \[F_{1,78} = 17.97, p < 0.0001\] but no significant main effect of post-injection time \[F_{4,78} = 0.77, p = 0.5480\]. There was also no significant interaction between challenge drug and post-injection time \[F_{4,78} = 0.77, p = 0.5480\]. A Newman-Keuls’ test indicated that tandospirone increased pAkt at Ser473 in the PVN over saline at 10 minutes (p < 0.05) post-injection.

2. **Experiment 2: Dose-dependent 5-HT\textsubscript{1A} receptor-mediated phosphorylation of Akt at Thr308 and Ser473 in the peripubertal hypothalamic PVN.** The objectives of this experiment were: to determine potency and efficacy of agonists from different structural drug classes ((+)8-OH-DPAT, an aminotetralin and tandospirone, an azapirone) on (A) phosphorylation of Akt at Thr308 and (B) phosphorylation of Akt at Ser473 in the PVN.
A. Effects of different doses of (+)8-OH-DPAT on levels of phosphorylated Akt at Thr308 in peripubertal rat hypothalamic PVN. As shown in figure 38, (+)8-OH-DPAT dose-dependently increased levels of pAkt (Thr308) over saline-challenged peripubertal rats. The phosphorylation of Akt at Thr308 induced by (+)8-OH-DPAT reached levels around 3.5 fold over basal.

The two-way ANOVA for (+)8-OH-DPAT-induced increases in pAkt (Thr308) in the PVN indicated a significant main effect of challenge drug (saline or (+)8-OH-DPAT) \( [F_{1,71} = 69.44, p < 0.0001] \) and a significant main effect of agonist dose \( [F_{3,71} = 12.24, p < 0.0001] \). There was also a significant interaction between challenge drug and agonist dose \( [F_{3,71} = 12.24, p < 0.0001] \). A Newman-Keuls’ test indicated that (+)8-OH-DPAT increased levels of pAkt (Thr308) in the PVN over saline at 0.10, 0.20, and 0.50 mg/kg (p < 0.01).

B. Effects of different doses of tandospirone on levels of phosphorylated Akt at Thr308 in peripubertal rat hypothalamic PVN. As shown in figure 39, most tested doses of tandospirone maximally increased levels of pAkt (Thr308) over saline-challenged peripubertal rats (to about 2.5 fold over basal). The maximal phosphorylation of Akt at Thr308 induced by (+)8-OH-DPAT was higher (~3.5 fold over basal) than tandospirone (~2.5 fold over basal). (+)8-OH-DPAT was also more potent in phosphorylating Akt at Thr308 (figure 38).

The two-way ANOVA for tandospirone-induced increases in pAkt (Thr308) in the PVN indicated a significant main effect of challenge drug (saline or
tandospirone) \( [F_{1,71} = 104.89, \ p < 0.0001] \) but no significant main effect of agonist dose (although there was a trend towards significance) \( [F_{3,71} = 2.46, \ p = 0.703] \). There was also no significant interaction (although a trend towards significance) between challenge drug and agonist dose \( [F_{3,71} = 2.46, \ p = 0.703] \). A Newman-Keuls’ test indicated that tandospirone increased levels of pAkt (Thr308) in the PVN over saline at 1 mg/kg \( (p < 0.05) \) and at 3, 10, and 20 mg/kg \( (p < 0.01) \).

C. Effects of different doses of \((+)-8\)-OH-DPAT on levels of phosphorylated Akt at Ser473 in peripubertal rat hypothalamic PVN. As shown in figure 40, \((+)-8\)-OH-DPAT dose-dependently increased levels of pAkt (Ser473) over saline-challenged peripubertal rats. \((+)-8\)-OH-DPAT increased the phosphorylation of Akt at Ser473 ~1.75 fold over basal.

The two-way ANOVA for \((+)-8\)-OH-DPAT-induced pAkt (Ser473) in the PVN indicated a significant main effect of challenge drug (saline or \((+)-8\)-OH-DPAT) \( [F_{1,71} = 20.55, \ p < 0.0001] \) and a significant main effect of agonist dose \( [F_{3,71} = 6.37, \ p = 0.0008] \). There was also a significant interaction between challenge drug and agonist dose \( [F_{3,71} = 6.37, \ p = 0.0008] \). A Newman-Keuls’ test indicated that \((+)-8\)-OH-DPAT at 0.50 mg/kg increased levels of pAkt (Ser473) in the PVN over saline \( (p < 0.01) \).

D. Effects of different doses of tandospirone on levels of phosphorylated Akt at Ser473 in peripubertal rat hypothalamic PVN. As shown in figure 41, most tested doses of tandospirone maximally increased levels of pAkt (Ser473) over
saline-challenged peripubertal rats (~1.4 fold over basal). (+)8-OH-DPAT was more potent than tandospirone for this response and had a higher maximal level of pAkt (Ser473) (~1.75 fold vs. ~1.4 fold over basal) (figure 40).

The two-way ANOVA for tandospirone-induced increases in pAkt (Ser473) in the PVN indicated a significant main effect of challenge drug (saline or tandospirone) \([F_{1,71} = 21.97, p < 0.0001]\) but no significant main effect of agonist dose \([F_{3,71} = 1.16, p = 0.3307]\). There was also no significant interaction between challenge drug and agonist dose \([F_{3,71} = 1.16, p = 0.3307]\). A Newman-Keuls’ test indicated that tandospirone at 3, 10, and 20 mg/kg increased levels of pAkt (Ser473) in the PVN over saline \((p < 0.05)\).

3. **Experiment 3: Determination of 5-HT\textsubscript{1A} receptor specificity for (+)8-OH-DPAT-mediated phosphorylation of Akt at Ser473.** The objective of this experiment was to administer a specific 5-HT\textsubscript{1A} receptor antagonist (WAY100635) to block receptor activation to determine if (+)8-OH-DPAT is acting specifically through 5-HT\textsubscript{1A} receptors (and not any other subtype of 5-HT receptors, e.g., 5-HT\textsubscript{7}) to induce increases of pAkt at Ser473 in the peripubertal PVN. 5-HT\textsubscript{1A} receptor-mediated phosphorylation at Ser473 was the focus of this experiment, since there is some evidence that the phosphorylation of Akt at Ser473 by the mTOR complex occurs prior to the recruitment of Akt to the plasma membrane and subsequent phosphorylation at Thr308 by its upstream kinase PDK1 (Sarbossov et al., 2005).
A. Effects of WAY100635 treatment on basal and 5-HT\textsubscript{1A} receptor-mediated increases in levels of pAkt (Ser473) in PVN. As shown in figure 42, (+)8-OH-DPAT significantly increased pAkt (Ser473) in the PVN by about 50% over saline. Treatment with the 5-HT\textsubscript{1A} receptor antagonist WAY100635 prior to challenge injection completely blocks (+)8-OH-DPAT-mediated increases in pAkt (Ser473) in the PVN. WAY100635 had no affect on basal levels of pAkt (Ser473) in the PVN.

The two-way ANOVA for levels of pAkt (Ser473) in the PVN indicated no main effect of WAY100635 treatment [F\textsubscript{1,25} = 2.73, p = 0.1129] and no significant main effect of challenge injection [F\textsubscript{1,25} = 3.39, p = 0.0791]. However, there was a significant interaction between treatment and challenge injection [F\textsubscript{1,25} = 7.02, p = 0.0147]. The Newman-Keuls’ test indicated that saline treatment/(+)8-OH-DPAT challenge significantly elevated pAkt (Ser473) in the PVN over the saline-treated/saline-challenged group (p < 0.05) and also that WAY100635 treatment completely blocked 5-HT\textsubscript{1A} receptor-induced increases in pAkt (Ser473) in the peripubertal PVN (p < 0.05).

4. Experiment 4: Determination of the role of PTX-sensitive G\alpha\textsubscript{i/o} proteins in 5-HT\textsubscript{1A} receptor-mediated phosphorylation of Akt at Ser473 in the peripubertal PVN. 5-HT\textsubscript{1A} receptor-mediated activation of Akt has been shown to be blocked with pertussis toxin (PTX) in hippocampal cell cultures (Cowen et al, 2005), indicating that this response may be mediated by PTX-sensitive G\alpha\textsubscript{i/o} proteins.
The objective of this experiment was to use PTX to functionally inactivate Gαi/o proteins (as previously demonstrated in adult rats, Garcia et al., 2006) to determine if this subset of proteins plays a role in 5-HT$_{1A}$ receptor-mediated increases of pAkt at Ser473 in the peripubertal PVN. Intra-PVN injection with PTX decreased levels of Gαi1 and Gαi2, increased levels of Gαo, and had no effect on levels of Gαi3 and Gαz in the peripubertal hypothalamus (Chapter 4).

5-HT$_{1A}$ receptor-mediated phosphorylation at Ser473 was the focus of this experiment, as there is some evidence that the phosphorylation of Akt at Ser473 by the mTOR complex occurs prior to the recruitment of Akt to the plasma membrane and subsequent phosphorylation at Thr308 by its upstream kinase PDK1 (Sarbossov et al., 2005).

A. Effects of PTX treatment on basal and 5-HT$_{1A}$ receptor-mediated increases in levels of pAkt (Ser473) in PVN. As shown in figure 43, (+)8-OH-DPAT increased pAkt (Ser473) in the PVN by about 50% over basal in vehicle-treated rats. In rats treated with PTX, however, 5-HT$_{1A}$ receptor-mediated increases in pAkt (Ser473) were not significantly elevated over its respective basal (↑24%). However, PTX also produced an increase in the basal levels of pAkt (Ser473) (↑23%) in the PVN that precludes any further increase by 5-HT$_{1A}$ receptor stimulation.

The two-way ANOVA for levels of pAkt (Ser473) in the PVN indicated no main effect of PTX treatment [$F_{1,37} = 0.28$, $p = 0.5998$] but a significant main effect of challenge injection [$F_{1,37} = 11.27$, $p = 0.0020$]. There was no significant
interaction between treatment and challenge injection \([F_{1,37} = 1.55, p = 0.2220]\). Although the basal levels of phosphorylated Akt (Ser473) in the PVN increased by about 23% with PTX treatment, this did not reach statistical significance, nor did 5-HT\(_{1A}\) receptor-stimulated elevations of pAkt in the PTX-treated group (\(\uparrow24\%\) over its respective basal and 45% over the vehicle-treated/saline-challenged group). The Newman-Keuls' test indicated that vehicle treatment/(+8-OH-DPAT challenge significantly elevated pAkt (Ser473) in the PVN over the vehicle-treated/saline-challenged group \((p < 0.05)\) and that PTX treatment/(+8-OH-DPAT challenge significantly elevated pAkt (Ser473) in the PVN over the vehicle-treated/saline-challenged group \((p < 0.05)\).

5. **Experiment 5: Determination of chronic FLX-induced effects on (A) basal and 5-HT\(_{1A}\) receptor-mediated increases of pAkt (Thr308) and (B) basal and 5-HT\(_{1A}\) receptor-mediated increases of pGSK3\(\beta\) in the peripubertal PVN.** Acute injection with fluoxetine has been shown to increase levels of pGSK3\(\beta\) in the mouse prefrontal cortex, hippocampus, and striatum, most likely due to the activation of 5-HT\(_{1A}\) receptors (Li et al., 2004). The objective of this experiment was to determine if chronic FLX injections (once per day for 7 or 14 days) have any effects on basal or 5-HT\(_{1A}\) receptor-mediated pAkt and basal or 5-HT\(_{1A}\) receptor-mediated pGSK3\(\beta\) responses in the peripubertal PVN. The activation of Akt via phosphorylation at Thr308 was measured, as (+)8-OH-DPAT induced a phosphorylation of Akt at Thr308 that was greater compared to Ser473 (see dose-
response experiment, experiment 2 of this chapter), and thus may be more sensitive to detect changes induced by chronic FLX.

A. Effects of FLX treatment on basal and 5-HT$_{1A}$ receptor-mediated increases in levels of pAkt (Thr308) in PVN. As shown in figure 44, in the rats treated for 7 days with saline (PD 35-41), (+)8-OH-DPAT increased pAkt (Thr308) in the PVN (↑313%), consistent with data from experiment 1 of this chapter. (+)8-OH-DPAT challenge after treatment for 14 days with saline (PD 28-41) increased pAkt (Thr308) in the PVN, although this response was not as robust (↑109%). For both 7D and 14D chronic treatments, FLX had no significant effect on (+)8-OH-DPAT-induced increases in pAkt (Thr308). There was also a non-significant increase (↑70%) in basal levels of pAkt (Thr308) with 7D FLX.

The two-way ANOVA for levels of pAkt (Thr308) in the PVN indicated no main effect of 7 days of FLX treatment [$F_{1,22} = 0.02, p = 0.8993$] but a significant main effect of challenge injection [$F_{1,22} = 48.25, p < 0.0001$]. There was a significant interaction between treatment and challenge injection [$F_{1,22} = 4.68, p = 0.0435$]. The Newman-Keuls’ test indicated that saline treatment/(+)8-OH-DPAT challenge significantly elevated pAkt (Thr308) in the PVN over the saline-treated/saline-challenged group ($p < 0.01$) and also that 7D FLX treatment/(+)8-OH-DPAT challenge significantly elevated pAkt (Thr308) in the PVN over the 7D FLX-treated/saline-challenged group. Although the basal levels of
phosphorylated Akt (Thr308) in the PVN increased by about 70% with 7 days of FLX treatment, this did not reach statistical significance.

The two-way ANOVA for levels of pAkt (Thr308) in the PVN indicated no main effect of 14 days of FLX treatment \([F_{1,23} = 0.04, p = 0.8389]\) but a significant main effect of challenge injection \([F_{1,23} = 9.25, p = 0.0064]\). There was no significant interaction between treatment and challenge injection \([F_{1,23} = 0.06, p = 0.8101]\). Although the (+)8-OH-DPAT increased levels of phosphorylated Akt (Thr308) in the PVN in both 14D saline-treated (↑109%) and 14D FLX-treated (↑92%), this did not reach statistical significance in the Neuman-Keuls post-hoc test.

B. Effects of FLX treatment on basal and 5-HT1A receptor-mediated increases in levels of pGSK3β in PVN. As shown in figure 45, (+)8-OH-DPAT increased pGSK3β in the PVN of rats treated for 7 days with saline (↑114%) and in rats treated for 14 days with saline (↑150%). Seven days of chronic FLX significantly attenuated (+)8-OH-DPAT-induced increases (to only 30% over basal) in pGSK3β, and 14D FLX partially attenuated (+)8-OH-DPAT-induced increases (to 80% over basal) in pGSK3β.

The two-way ANOVA for levels of pGSK3β in the PVN indicated no main effect of 7 days of FLX treatment \([F_{1,23} = 2.26, p = 0.1480]\) but a significant main effect of challenge injection \([F_{1,23} = 10.35, p = 0.0043]\). There was a trend towards a significant interaction between treatment and challenge injection \([F_{1,23} = 3.29, p\)
The Newman-Keuls’ test indicated that saline treatment/(+)8-OH-DPAT challenge significantly elevated pGSK3β in the PVN over the saline-treated/saline-challenged group (p < 0.01). Seven days FLX treatment significantly attenuated increases in pGSK3β induced by (+)8-OH-DPAT challenge compared to chronic saline treatment (p < 0.05).

The two-way ANOVA for levels of pGSK3β in the PVN indicated no main effect of 14 days of FLX treatment [F_{1,23} = 1.48, p = 0.2384] but a significant main effect of challenge injection [F_{1,23} = 20.68, p = 0.0002]. There was no significant interaction between treatment and challenge injection [F_{1,23} = 1.77, p = 0.1984]. The Newman-Keuls’ test indicated that (+)8-OH-DPAT challenge significantly elevated pGSK3β in the PVN over the saline challenge in both the 14D saline-treated (p < 0.01) and 14D FLX-treated (p < 0.05) groups.
Figure 34. (A) Time-course of (+)8-OH-DPAT-induced elevations of pAkt at Thr308 in peripubertal rat hypothalamic PVN. *, ** indicates significant difference (p < 0.05 and p < 0.01, respectively) from the saline-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 7-8 per group. (B) Representative western blot of pAkt (Thr308) (above) and Akt (below) from the PVN of rats treated with either saline or (+)8-OH-DPAT.
Figure 35. (A) Time-course of tandospirone-induced elevations of pAkt at Thr308 in peripubertal rat hypothalamic PVN. * indicates significant difference (p < 0.05) from the saline-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 7-8 per group. (B) Representative western blot of pAkt (Thr308) (above) and Akt (below) from the PVN of rats treated with either saline or tandospirone.
Figure 36. (A) Time-course of (+)-8-OH-DPAT-induced increases in phosphorylated Akt at Ser473 in the peripubertal rat hypothalamic PVN. *, ** indicates significant difference (p < 0.05 and p < 0.01, respectively) from the saline-challenged group; + indicates significant difference (p < 0.05) from 5 minute (+)-8-OH-DPAT-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 8 per group. (B) Representative western blot of pAkt (Ser473) (above) and Akt (below) from the PVN of rats treated with either saline or (+)-8-OH-DPAT.
Figure 37. (A) Time-course of tandospirone-induced increases in phosphorylated Akt at Ser473 in the peripubertal rat hypothalamic PVN. ** indicates significant difference (p < 0.01) from the saline-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 7-8 per group. (B) Representative western blot of pAkt (Ser473) (above) and Akt (below) from the PVN of rats treated with either saline or tandospirone.
Figure 38. (A) Effects of different doses of (+)8-OH-DPAT on phosphorylation of Akt at Thr308 in the peripubertal rat hypothalamic PVN at 10 minutes post-injection. ** indicates significant difference (p < 0.01) from the saline-challenged group; +, ++ indicates significant difference (p < 0.05 and p < 0.01, respectively) from the 0.04 mg/kg (+)8-OH-DPAT-challenged group; ## indicates significant difference (p < 0.01) from the 0.10 mg/kg (+)8-OH-DPAT-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 6-12 per group. (B) Representative western blot of pAkt (Thr308) (above) and Akt (below) from the PVN of rats treated with either saline or (+)8-OH-DPAT.
Figure 39. (A) Effects of different doses of tandospirone on phosphorylation of Akt at Thr308 in the peripubertal rat hypothalamic PVN at 10 minutes post-injection. *, ** indicates significant difference (p < 0.05 and p < 0.01, respectively) from the saline-challenged group; +, ++ indicates significant difference (p < 0.05 and p < 0.01, respectively) from the 1 mg/kg tandospirone-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 6-12 per group. (B) Representative western blot of pAkt (Thr308) (above) and Akt (below) from the PVN of rats treated with either saline or tandospirone.
Figure 40. (A) Effects of different doses of (+)8-OH-DPAT on the phosphorylation of Akt at Ser473 in the peripubertal rat hypothalamic PVN at 10 minutes post-injection. ** indicates significant difference (p < 0.01) from the saline-challenged group; ++ indicates significant difference (p < 0.01) from the 0.04 mg/kg (+)8-OH-DPAT-challenged group; ## indicates significant difference (p < 0.01) from the 0.10 mg/kg (+)8-OH-DPAT-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 6-12 per group. (B) Representative western blot of pAkt (Ser473) (above) and Akt (below) from the PVN of rats treated with either saline or (+)8-OH-DPAT.
Figure 41. (A) Effects of different doses of tandospirone on the phosphorylation of Akt at Ser473 in the peripubertal rat hypothalamic PVN at 10 minutes post-injection. * indicates significant difference (p < 0.05) from the saline-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 6-12 per group. (B) Representative western blot of pAkt (Ser473) (above) and Akt (below) from the PVN of rats treated with either saline or tandospirone.
Figure 42. (A) The effects of either saline or the 5-HT\textsubscript{1A} receptor antagonist WAY100635 on basal and 5-HT\textsubscript{1A} receptor-mediated increases in pAkt (Ser473) in the peripubertal rat hypothalamic PVN. * indicates significant difference (p < 0.05) from the respective saline-challenged group; # indicates significant difference (p < 0.05 from the saline treated/(+)8-OH-DPAT challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 6-8 per group. (B) Representative western blot of pAkt (Ser473) (above) and Akt (below) from the PVN of rats treated with saline or WAY100635 and subsequently challenged with either saline or (+)8-OH-DPAT.
Figure 43. (A) The effects of intra-PVN injection with either vehicle or pertussis toxin (PTX) on basal and 5-HT1A receptor-mediated increases in pAkt (Ser473) in the peripubertal rat hypothalamic PVN. * indicates significant difference (p < 0.05) from the respective saline-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 6-12 per group. (B) Representative western blot of pAkt (Ser473) (above) and Akt (below) from the PVN of rats treated with vehicle or PTX and subsequently challenged with either saline or (+)8-OH-DPAT.
Figure 44. The effects of (A) 7 days and (B) 14 days of chronic FLX injections on basal and 5-HT$_{1A}$ receptor-mediated increases in pAkt (Thr308) in the peripubertal rat hypothalamic PVN. ** indicates significant difference (p < 0.01) from the respective saline-challenged group (two-way ANOVA and Newman-Keuls multiple-range test). N = 5-6 per group. (C) Representative western blot of pAkt (Thr308) (above) and Akt (below) from the PVN of rats treated with sal or FLX and subsequently challenged with either saline or (+)8-OH-DPAT. 7D treatment is on the left and 14D treatment is on the right.
Figure 45. The effects of (A) 7 days and (B) 14 days of chronic FLX injections on basal and 5-HT$_{1A}$ receptor-mediated increases in pGSK3β in the peripubertal rat hypothalamic PVN. *, ** indicates significant difference (p < 0.05 and p < 0.01, respectively) from the respective saline-challenged group; # indicates significant difference (p < 0.05) from the respective vehicle-treated group (two-way ANOVA and Newman-Keuls multiple-range test). N = 6 per group. (C) Representative western blot of pGSK3β (above) and GSK3β (below) from the PVN of rats treated with sal or FLX and subsequently challenged with either saline or (+)-8-OH-DPAT. 7D treatment is on the left and 14D treatment is on the right.
Stressors suffered during childhood and adolescence (e.g., bullying, home displacement as with Hurricane Katrina, and abuse) and mental disorders such as depression have all been linked to hypothalamic dysfunction (Vaillancourt et al., 2007; Lopez-Duran et al., 2009; Vigil et al., 2010; Hankin et al., 2010). Preclinical studies have determined that, in a rat model of chronic stress, levels of pAkt were increased in the hypothalamus, hippocampus, and striatum, with the largest increases observed in the hypothalamus (Lee et al., 2006). 5-HT$_{1A}$ receptor agonists have been shown to increase levels of phosphorylated Akt and its downstream effectors in vitro (Adayev et al., 1999; Cowen et al., 2005; Hsiung et al., 2005) and in vivo (Polter et al., 2010). The studies described in this chapter provide the first demonstration that 5-HT$_{1A}$ receptors can activate Akt via phosphorylation of Thr308 and Ser473 in the rat hypothalamus, and the first to characterize this signal transduction pathway and its regulation by fluoxetine in the peripubertal PVN.

Our data reveal that the agonist (+)8-OH-DPAT mediates phosphorylation of Akt specifically via 5-HT$_{1A}$ receptors in the peripubertal PVN. (+)8-OH-DPAT elevated levels of pAkt at Ser473 in the PVN, which was completely blocked by pretreatment with the 5-HT$_{1A}$ receptor antagonist WAY100635. Recent evidence reveals that phosphorylation of Akt at Ser473 (within the hydrophobic motif of the protein) may promote recruitment of Akt to the plasma membrane and
enhances the interaction between Akt and PDK1, leading to the subsequent phosphorylation of Akt at Thr308 (Sarbossov et al., 2005). Therefore, we did not measure levels of pAkt Thr308 in this experiment but, considering that phosphorylation of Akt at Ser473 most likely precedes that of Thr308, we would expect that (+)8-OH-DPAT-mediated increases in pAkt at Thr308 (as measured in other experiments in this chapter) is primarily by 5-HT1A receptors.

Both Thr308 and Ser473 must be phosphorylated in order to fully activate Akt (Alessi et al., 1996). Each of these sites is phosphorylated by different (and most likely independently regulated) upstream kinases: PDK1 phosphorylates Thr308 (Alessi et al., 1997; Anderson et al., 1998; Stephens et al., 1998), and the rictor/mTOR complex has recently been identified as the kinase for Ser473 (Sarbassov et al., 2005). We have shown that injection with two different 5-HT1A receptor agonists, (+)8-OH-DPAT and tandospirone, resulted in a rapid phosphorylation of Akt at both Thr308 and Ser473, although the magnitude of phosphorylation varied with agonist. (+)8-OH-DPAT induced a higher maximal effect and was more potent than tandospirone in phosphorylating Akt at both Thr308 and Ser473. These data indicate that (+)8-OH-DPAT is a “full” agonist and tandospirone a “partial” agonist in 5-HT1A receptor-mediated phosphorylation of Akt in the peripubertal PVN. Therefore, (+)8-OH-DPAT may more effectively couple to the pathway initiating PDK1 phosphorylation of Akt
at Thr308 and the raptor/mTOR-mediated phosphorylation of Akt at Ser473 than tandospirone.

A previous study had shown that 5-HT$_{1A}$ receptor-mediated activation of Akt in hippocampal cell cultures can be blocked with pertussis toxin (PTX) treatment (Cowen et al., 2005), indicating that 5-HT$_{1A}$ receptor-mediated activation of Akt could be dependent on PTX-sensitive G$\alpha$/$\beta$ proteins. In experiment 4 of this chapter, we determined that intra-PVN injection with PTX prevented significant elevations of 5-HT$_{1A}$ receptor-mediated phosphorylation of Akt at Ser473. Therefore, since G$\alpha_{11}$ and G$\alpha_2$ proteins were the only G$\alpha$ proteins reduced with PTX treatment (Chapter 4), these proteins may be playing a role in 5-HT$_{1A}$ receptor-mediated phosphorylation of Akt. However, considering that PTX treatment also increased basal levels of phosphorylated Akt at Ser473, this could have precluded obtaining a significant (+)8-OH-DPAT-induced increase above basal. Therefore, we cannot rule out the possibility that 5-HT$_{1A}$ receptors may be signaling via G$\alpha_3$, G$\alpha_0$, or G$\alpha_z$ to mediate the phosphorylation and activation of Akt in the peripubertal PVN. Additionally, the PTX-induced increase in hypothalamic G$\alpha_0$ proteins may produce the elevations in basal pAkt (Ser473). Alternatively, 5-HT$_{1A}$ receptor-mediated Akt activation could be mediated by a mechanism other than one mediated by G$\alpha$ proteins. Akt can directly associate with G$\beta$$_Y$ subunits in rat brain extracts (Konishi et al., 1995). Akt can also be
activated in a G protein-independent, β-arrestin dependent manner, as has been demonstrated in fibroblast cell lines (Goel et al., 2002; Povsic et al., 2003).

Akt activation leads to an increase in phosphorylated GSK3β, which inactivates this constitutively active protein kinase (Cross et al., 1995). In mice, acute injection with fluoxetine increases levels of pGSK3β in the mouse prefrontal cortex, hippocampus, and striatum, which is most likely due to a synaptic increase in 5-HT and subsequent activation of 5-HT1A receptors (Li et al., 2004). However, it was not known if the 5-HT1A receptor-mediated activation of the Akt pathway in the peripubertal PVN was subject to regulation upon chronic treatment with FLX. We determined that 5-HT1A receptor-mediated phosphorylation of Akt is generally not affected with either 7 or 14 days of FLX. However, 14 days of injections with either saline or FLX rendered the animals hyporesponsive to the effects of (+)8-OH-DPAT on 5-HT1A receptor-mediated phosphorylation of Akt. This effect was also observed with the 5-HT1A receptor-mediated activation of ERK (Chapter 4). There may be stress-related effects upon an additional 7 days of injection. Alternatively, starting injections at PD 28 (as with the 14 day treatment paradigm used) as opposed to PD 35 (as with the 7 day treatment paradigm used) may have exposed the animals to injection stress at a critical point in postnatal development. Although we did not observe any specific FLX-induced effects on 5-HT1A receptor-mediated activation of Akt, its downstream target GSK3β was differentially affected by the 7 and 14 day chronic
treatments. There was no hyporesponsive effect of 14 days of injection (as observed with (+)8-OH-DPAT challenge on pERK and pAkt) in either saline or FLX-treated animals on phosphorylated levels of GSK3β. We observed almost a complete desensitization of (+)8-OH-DPAT-induced increases in pGSK3β with 7 days FLX treatment. With 14 days of FLX treatment, this desensitization is not as marked. Either the levels of pGSK3β elicited by (+)8-OH-DPAT were starting to recover or, alternatively, starting treatment earlier in postnatal development was not having a robust effect on (+)8-OH-DPAT-stimulated pGSK3β. FLX could possibly be modulating the protein levels or activity of a pGSK3β-specific phosphatase, which may account for the differential effects on GSK3β but not Akt phosphorylation. Duric et al. determined that chronic stress increases hippocampal levels of a phosphatase specific for MAPK, and that antidepressants normalize the levels of this phosphatase (Duric et al., 2010). More closely linked with the Akt/GSK3β signaling pathway, protein levels of PP1 phosphatase, the primary phosphatase for GSK3β in COS-7 cell cultures (Hernandez et al., 2010), were increased in the frontal cortex and hippocampus of adult rats subjected to a model of depression compared to sham rats (Feng et al., 2003). Therefore, chronic FLX may be regulating the protein levels or activity of phosphatases specific to GSK3β.

Together, these data provide the first in vivo demonstration that 5-HT1A receptors can activate Akt in the peripubertal PVN. Given the previous studies
demonstrating that 5-HT$_{1A}$ receptors can stimulate hormone responses and activate ERK activation in the peripubertal PVN, these findings are the first to show that 5-HT$_{1A}$ receptors can mediate the concomitant activation of three signaling pathways in peripubertal hypothalamic PVN and that chronic FLX can regulate downstream targets of 5-HT$_{1A}$ receptor-mediated Akt pathways in the peripubertal PVN in vivo.
CHAPTER 7

DETAILED METHODS

ANIMALS

Male Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN). The determination of post-natal day zero (day of birth) was carried out by the supplier. All animals had 2-3 days to acclimate to their home cage and were housed two per cage in a room controlled for temperature (20-22°C), humidity (50-55%), and illumination (12h: 12h light/dark cycle; lights on at 07:00 h). Food and water were available ad libitum. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as approved by Loyola University Institutional Animal Care and Use Committee (IACUC). Every effort was made to minimize suffering and discomfort to the animals. The number of rats per group was the minimum needed to obtain statistically meaningful evaluation of the data. Our power analysis and past experience indicate that a minimum of 8 rats per group is needed to obtain hormone data that can be analyzed statistically because ACTH and oxytocin are “stress hormones” (Jezova et al., 1993; Jezova et al., 1996) and some rats may be more sensitive to stress effects of handling and injection.
HORMONE DETERMINATIONS

**Blood collection and centrifugation.** After sacrifice, trunk blood was collected in centrifuge tubes containing 0.3 M ethylenediamine tetraacetic acid solution (EDTA, pH 7.4; 0.25 ml for peripubertal or prepubertal rats and 0.50 ml for adult rats). The blood was centrifuged at 3000 x g for 40 minutes at 4°C and the plasma was divided into aliquots. Plasma aliquots were stored at -80°C until the respective hormones were determined by radioimmunoassay.

**Plasma oxytocin radioimmunoassay.** Plasma levels of oxytocin were determined by a double antibody assay using methods modified from Li et al. (1997). First, plasma was extracted the day before the start of the assay. Rat plasma (0.80 ml) was mixed with 1.6 ml ice cold acetone and centrifuged at 1600xg, at 4°C for 30 min. Then, the supernatant was added to 4 ml ice cold petroleum ether and thoroughly mixed by inverting the tube several times. This mixture was centrifuged at 1600xg, at 4°C for 15 min. The top layer was aspirated and discarded and the remaining solution was dried by blowing air into the tubes at 4°C. A known quantity of oxytocin standard was added to several tubes of normal rat plasma; these tubes were used to determine the efficiency of the extraction. The tubes containing the dried extract were stored overnight at 4°C.

On the day of the assay, the dried extract was reconstituted in 0.80 ml of assay buffer (0.05 M phosphate buffer, pH 7.4, containing 0.125% bovine serum
The reconstituted plasma extract (100 μl or 200 μl, each in duplicate) and oxytocin standards (Bachem Fine Chemicals, Torrance, CA) were incubated with 0.1 ml of rabbit anti-oxytocin serum (1:5,000 dilution) in a total volume of 0.4 ml for 24 hr at 4°C. 125I-oxytocin (Du Pont-NEN, Boston, MA, 3000 cpm, 0.1 ml) was then added to the tubes and incubated for 72 hr at 4°C. Next, 0.1 ml goat anti-rabbit γ-globulin (1:20 dilution) was added to each of the tubes, followed by 0.1 ml of normal rabbit serum (1:120 dilution). After incubation for 24 hr, the tubes were centrifuged at 2300xg, at 4°C for 20 min. The supernatant was decanted, and the radioactivity in the pellet was counted for 5 min by a Micromedic 4/200 plus γ counter and analyzed from the standard curve using the RIA-AID computer program (Robert Maciel Associates, Arlington, MA). The concentration of plasma oxytocin was calculated with a correction factor based on the recovery of the extraction. The sensitivity limit of this assay is 1 pg/tube, and the intra- and interassay variabilities are 8.1% and 8.6%, respectively.

**Plasma ACTH radioimmunoassay.** This assay is a double-antibody assay and was performed as previously described (Van de Kar et al., 1989). Briefly, plasma from each rat (unextracted plasma samples, 10 μl and 50 μl each in duplicate) and ACTH standards (Calbiochem, Los Angeles, CA) were incubated with 0.1 ml of rabbit anti-ACTH antibody (IgG Corp, Nashville, TN) in a total volume of 0.2 ml for 24 hr at 4°C. 125I-ACTH tracer (DiaSorin, Inc., Stillwater, MN)
was then added to the tubes and incubated for 48 hr at 4°C. Next, 0.1 ml goat anti-rabbit γ-globulin (1:20 dilution) was added to each of the tubes. After incubation for 24 hr, 1.5 ml cold PBS buffer (0.01 M phosphate buffer + 0.15 M NaCl, pH 7.6) were added to each of the tubes. The tubes were then centrifuged at 2300 x g, at 4°C for 40 min. The supernatant was decanted, and the radioactivity in the pellet was counted for 5 min by a Micromedic 4/200 plus γ counter and analyzed from the standard curve using the RIA-AID computer program (Robert Maciel Associates, Arlington, MA). The sensitivity limit of this assay is 0.25 pg/tube, and the intra- and inter-assay variability is 4.2% and 14.6%, respectively (Van de Kar et al., 1989).

**Plasma testosterone radioimmunoassay.** This assay is a solid-phase radioimmunoassay, based on testosterone-specific antibody immobilized to the wall of a polypropylene tube. The Coat-A-Count® Total Testosterone kit was purchased from Siemens (Los Angeles, CA). Briefly, plasma from each rat (50 μl, in duplicate) was placed in an antibody-coated tube and incubated with 1.0 ml of 125I-testosterone for 3 hours at 37°C with light shaking. After this incubation, the supernatant was decanted, and the radioactivity in the pellet was counted for 3 min by a Micromedic 4/200 plus γ counter and analyzed from the standard curve using Microsoft Excel. The sensitivity limit of this assay is 4 ng/dl, and the intra- and inter-assay variability is 7.3% and 11.0%, respectively.
WESTERN BLOTTING

**Microdissection of brain areas of interest.** Brains were placed in a cryostat at -12°C. A 650 μm coronal section including the PVN was cut from each brain. This section was placed on a glass slide and adhered onto slides with Tissue Tek. The slides were then placed on a large deep Petri dish on an ice/salt mixture (to maintain the temperature at -5°C). The hypothalamic PVN was dissected from its slice as a triangular “punch” (Crane et al., 2007; Serres et al., 2000a).

In the pertussis toxin experiment (Chapter 4), brains from each of the treatment groups (vehicle and PTX) had the whole hypothalamus dissected immediately after the brain was removed from the skull. Each whole hypothalamus was placed in a tube and immediately frozen in liquid nitrogen.

In the developmental Gα protein study (Chapter 4), the frontal cortex was removed from each frozen brain, and the olfactory bulbs were separated from cortical tissue and discarded. An additional 1100 μm coronal section including the hippocampus was also cut after the PVN section. The hippocampus was removed from its respective coronal section with the use of a small angular blade. Each of these respective brain regions were placed in individual 1.5 ml eppendorf tubes and stored at -80°C until prepared for western analysis.

**Tissue preparation for the determination of pERK/ERK, pAkt/Akt, and pGSK3β/GSK3β.** The tissue preparation was performed using methods modified from Crane et al. (2007), and all procedures were conducted at 4°C
unless otherwise noted. Briefly, the PVN was homogenized in 10 mM Tris buffer (pH 7.6) containing 100 mM NaCl, 1 mM EDTA, 2 mM activated sodium orthovanadate, and 50 mM NaF with additional protease inhibitors purchased in a cocktail (1 μg/10 mg of tissue; Sigma, St. Louis, MO). This homogenate was stored at -80°C until ready for Western blot analysis. A bicinchoninic acid (BCA) assay (Pierce Chemical, Rockford, IL) was performed to quantify total protein as per the manufacturer’s microplate procedure.

**Tissue preparation for the determination of the levels of Ga proteins.** All procedures were conducted at 4°C unless otherwise indicated. The tissues (PVN, hippocampus, frontal cortex, or whole hypothalamus) were homogenized in 50 mM Tris buffer (pH 7.7) containing 150 mM NaCl, 10% sucrose, 0.5 mM phenylmethanesulfonyl fluoride (PMSF) and additional protease inhibitors purchased as a cocktail (1 μl/30 mg of tissue; Sigma, St. Louis, MO). After centrifugation at 20,000 x g for 60 min, the supernatant was collected, aliquoted, and stored at -80°C for Western blot analyses of cytosol-associated protein levels. The pellets (containing the membrane-associated proteins) were solubilized and resuspended in 20 mM Tris buffer (pH 8), containing 1 mM EDTA, 100 mM NaCl, 1% sodium cholate, and 1 mM dithiothreitol, plus the protease inhibitory cocktail (1 μl/30 mg of tissue). The resuspended homogenates were placed on a shaker for 1 h at 4°C, followed by centrifugation at 40,000 x g for 60 min. The supernatant was collected, aliquoted, and stored at -80°C for Western blot
analyses of membrane-associated protein levels. Protein concentrations were measured using a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical, Rockford, IL) (Li et al., 1996).

**Separation of proteins using electrophoresis.** This assay was performed using methods modified from Li et al. (1996) and Crane et al. (2007). Protein (1-7 \( \mu \text{g} \) per lane) was loaded on 12.5% polyacrylamide gel and separated by electrophoresis (200 constant volts, 60-70 minutes). Proteins were subsequently electrophoretically transferred onto a nitrocellulose membrane (100 constant volts, 50-60 minutes). After transfer, the nitrocellulose membranes were incubated for 1 hour at room temperature on a shaker in a blocking solution of 0.1 M phosphate-buffered saline (PBS) containing 5% bovine serum albumin and 0.1% Tween-20 when probing for phosphorylated proteins or 0.1 M PBS containing 0.2% I-block (Tropix, Inc.) and 0.05% Tween-20 when probing for non-phosphorylated proteins. The blot was then incubated in primary antibody overnight at 4°C on a shaker (with an intermediate washing step of 2 x 2 minutes with 0.1 M PBS with 0.1% Tween-20 for phosphorylated proteins). Primary antibodies (from Cell Signaling: pERK, ERK, pAkt (Ser473), pAkt (Thr308), Akt, pGSK3\( \beta \), and GSK3\( \beta \); from Santa Cruz: G\( \alpha \)z and G\( \alpha \)i1; from Upstate: G\( \alpha \)i3/o; from Chemicon: G\( \alpha \)i2; and from ICN Biomedicals: actin) were diluted in the same blocking solution used. The blots were then washed (2 x 5 minutes, 1 x 10 minutes) in 0.1 M PBS with 0.1% Tween-20 and subsequently incubated in goat
anti-rabbit-HRP-conjugated (for pERK, ERK, Gαi3/o, and Gαz) or goat anti-mouse-HRP-conjugated (for GααiL, Gαi2, and actin) for 1 hour at room temperature on a shaker. HRP-labeled protein bands were detected with ECL (Amersham Chemical Co.) and captured on film as per the manufacturer’s protocol (Crane et al., 2007; Li et al., 1996).

**Immunoblot film analysis.** Films were analyzed densitometrically using Scion Image software (Scion Corporation, Frederick, MD). The gray-scale density readings were calibrated using a transmission step-wedge standard. The integrated optical density (IOD) of each band was calculated as the sum of optical densities of all the pixels within the area of the band outlined. An area adjacent to the outlined protein band was used to calculate the background optical density of the film and was subtracted from the IOD for each band. To control for inter-gel variability, the resulting IOD for each protein band was then divided by the average IOD from the control group (i.e., saline-challenged) protein bands contained in the same blot. Since tissue samples were measured on three independent gels, the mean “% of control” obtained from the three gels represent the data for each rat. The mean % of control for phosphorylated ERK, Akt, GSK3β, or Ga for each animal was divided by the mean % of control for the respective non-phosphorylated or actin protein level to control for differences in gel loading (modified from Crane et al., 2007).
For the pertussis toxin experiment, rats were anesthetized with a mixture of ketamine and xylazine (100 mg ketamine plus 7 mg xylazine per ml, 1.4 ml/kg, i.p.) and a double-barreled guide cannula (26 gauge, 1.2 mm center-to-center distance, Plastics One, Roanoke, VA) with its corresponding dummy cannula inserted inside was implanted into the brain above both sides of the paraventricular nucleus at the following stereotaxic coordinates: 1.6 mm caudal with respect to bregma, -0.6 mm lateral with respect to lambda, and 6.0 mm ventral from the skull surface. All cannula implantations were performed on rats that were at least PD 31. Rats were allowed to recover for several days before further treatment. Administration of PTX was performed according to procedures previously established in adult rats (Garcia et al., 2006). An injection cannula was inserted through the implanted guide cannula above the PVN, and rats received an intracerebral injection of vehicle (100 mM sodium phosphate + 500 mM NaCl, 0.5 μl/side) or PTX (1 μg/μl, 0.5 μl/side) over 1 minute. The injection cannula was left in situ for an additional 1 minute before removal. The brains were visually inspected during dissection to ensure proper implantation of the cannula.

**IMMUNOHISTOCHEMISTRY**

**Immersion Fixation of Brain Tissue.** This protocol was adapted from Khan and Watts, 2004. Immediately after harvest, brains were placed in ice-cold
sodium acetate-buffered 4% paraformaldehyde (pH 6.5) and were then stored at 4°C. After 24 hours, each brain was removed from the fixative, gently blotted dry, and placed in sodium borate-buffered 4% paraformaldehyde (pH 9.5) at 4°C for 48 hours. Brains were then transferred to 0.1 M sodium phosphate buffered 4% paraformaldehyde (pH 7.4) and 20% glycerol at 4°C for 1-2 days (until brains had sunk to the bottom of the container). Sections were cut on a sliding microtome in the coronal plane at 30 μm and stored as serial sets of one section in every twelve in ethylene-glycol based cryoprotectant solution and stored at -20°C until processed.

**Colocalization of Oxytocin and pERK.** For double-labeling for oxytocin and pERK, labeling protocols with immunofluorescence detection were adapted from Garcia-Ovejero et al., 2002; Khan and Watts et al., 2004; Khan et al., 2007; Lorenz et al., 2005. All washes and incubations were performed on free floating sections with shaking at room temperature unless otherwise indicated. First, brain sections were rinsed 3 times for 10 minutes each with Tris-buffered saline (TBS) (50 mM Tris and 150 mM NaCl, pH 7.4). The slices were then transferred to TBS containing 0.3% Triton X-100 for 50 minutes to permeabilize the sections. Sections were then incubated for 72 h at 4°C with anti-oxytocin rabbit polyclonal antiserum (Calbiochem) at a concentration of 1:1000 and mouse anti-phospho-p44/42 MAPK (ERK1/2) monoclonal antiserum (Cell Signaling) at a concentration of 1:800 in antibody solution (TBS + 2% normal goat serum).
Sections were then rinsed three times for 5 min each in TBS. Next, the slices were incubated with a combination of secondary antibodies for 2 hours at RT: Invitrogen goat anti-rabbit conjugated to Alexa568 (1:200) and Vector biotinylated goat anti-mouse (1:250) in secondary solution (TBS + 2% normal goat serum + 1% Triton X-100). After three washes of 10 min each (TBS), the slices were incubated with streptavidin conjugated to Alexa488 (Invitrogen; 1:200 in TBS) for 2 hours at RT. Lastly, the slides were washed again with TBS for three times 10 min each and two times 15 min each. Sections were mounted on gelatin-coated slides, allowed to dry for 30-40 minutes and then coverslipped with Fluoro-gel (Electron Microscopy Science #17985) and subsequently stored in a foil-wrapped box at 4°C. The sections were allowed to dry thoroughly and were later analyzed using an Olympus IX-81 fluorescent microscope. Additionally, some slides were analyzed with a confocal microscope (Zeiss LSM510) at a higher magnification to confirm localization of fluorescent markers.

DATA ANALYSIS

The data are represented as group means and the standard errors of the means (S.E.M.). GB-STAT software (Dynamic Microsystems, Inc.) was used for all statistical analyses.

For the immunohistochemistry experiment, to quantify the number of PVN neurons labeled for oxytocin or 5-HT₁A receptor-stimulated pERK, two sections from each rat were selected for analysis. These sections corresponded in plane
and rostrocaudal position most closely to that represented in plate 26 of Paxinos and Watson, 1998. For each fluorophore (Alexa488 and Alexa568), a stack of 30 images (1 μm per image) was obtained for each half of the PVN using an Olympus IX-81 fluorescent microscope. Each 1 μm-thick image was deconvolved using MicroTome Deconvolution 7.0 (VayTek, Fairfield, IA) and compressed into one composite image by ImagePro v6.3 (Scitech, Preston, Victoria, Australia). Therefore, 4 composite images were obtained for each rat for each fluorophore. Using Image J (NIH), each composite image was inverted so that the cell labeling for either oxytocin or pERK appeared as shades of gray on a white background. Then, optical density for each image was determined by first calculating the density of the shaded pixels in an 850 pixel by 950 pixel area (encompassing either the left or right half of the PVN), which was then compared to a calibrated optical density step tablet. The integrated density is defined as the product of the pixel area and the optical density. Also, the total cell count for each composite image was obtained using particle analysis in the ImageJ program. Therefore, the cell density is defined as the number of particles within the 850 x 950 pixel area for each composite image. The average of the integrated density and cell density was calculated from the values for each composite image (4 per rat). Confocal images were processed using Zeiss LSM Image Browser and ImageJ.
CHAPTER 8

GENERAL DISCUSSION

The serotonin 1A (5-HT$_{1A}$) receptor was one of the first serotonin receptor subtypes to be investigated with respect to its pharmacological functions and regional distribution in brain. Although the somatodentritic 5-HT$_{1A}$ autoreceptor population has received much attention due to its involvement in the therapeutic efficacy of various drugs used to treat mood disorders, postsynaptic 5-HT$_{1A}$ receptors on non-serotonergic cells may also be involved with antidepressant-induced neuroadaptations. Post-synaptic 5-HT$_{1A}$ receptors are localized in various brain regions such as the hippocampus, frontal cortex, and hypothalamus (Kia et al., 1996a; Kia et al., 1996b; Blier and Ward 2003; Marvin et al., 2010), where they can initiate a variety of signal transduction cascades (Raymond et al., 2001). 5-HT$_{1A}$ receptors in the hypothalamus are involved in the serotonergic stimulation of various hormones and regulation of the hypothalamic-pituitary-adrenal (HPA) axis. Recent studies have also shown that hypothalamic 5-HT$_{1A}$ receptors can activate extracellular signal regulated kinase (ERK) (Sullivan et al., 2005; Crane et al., 2007), a protein kinase involved with many functions including gene expression, mitosis, differentiation, cell survival/apoptosis, learning, memory, neuroplasticity, and receptor
desensitization (Marshall 1995; Pouysségur and Lenormand 2003; Trincavelli et al., 2002). However, the majority of information on 5-HT$_{1A}$ receptor signaling has been obtained from in vitro studies in cells or in vivo studies in adult animal models. Similarly, studies on the mechanisms of 5-HT$_{1A}$ receptor signaling in vivo in hypothalamic paraventricular nucleus (PVN) have also utilized adult animal models. Prior to these dissertation studies, there have been no comparable studies on 5-HT$_{1A}$ receptor signaling in the peripubertal hypothalamic PVN. Considering the significant contribution of the serotonergic system over brain development around the time of sexual maturation (Whitaker-Azmitia 2001), the role of serotonin in regulating neuroendocrine function, and that serotonergic modulation (e.g. with antidepressant treatment) produces responses that are distinct and more long-lasting than those produced in adults (Maciag et al., 2006; Ansorge et al., 2004; Taravosh-Lahn et al., 2006; Oh et al., 2009), this dissertation investigated 5-HT$_{1A}$ receptor-mediated signaling pathways in the hypothalamic PVN of peripubertal rats.

Previous studies demonstrated, in the adult rat hypothalamic PVN, 5-HT$_{1A}$ receptors activate independent signaling pathways (utilizing different Ga protein subtypes): G$_{az}$ proteins exclusively mediate the 5-HT$_{1A}$ receptor-induced increase in plasma levels of oxytocin and adrenocorticotropin hormone (ACTH) (Serres et al., 2000a) and concomitantly activate the mitogen-activated protein kinase (MAPK) ERK via pertussis toxin-sensitive G$_{ai/o}$ proteins (Crane
et al., 2007; Garcia et al., 2006; Sullivan et al., 2005). These pathways are independent, as blockade of ERK signaling does not alter 5-HT\textsubscript{1A} signaling of hormone responses (Jia et al., 2007).

The present studies provide the first \textit{in vivo} evidence that 5-HT\textsubscript{1A} receptors in the peripubertal PVN mediate the concomitant activation of multiple hormone and kinase signaling responses. More specifically, these dissertation studies demonstrated that 5-HT\textsubscript{1A} receptors stimulate increases in plasma levels of oxytocin and ACTH, phosphorylation of ERK, and phosphorylation of protein kinase B (Akt). We have also determined that G\textalpha{} proteins may be uniquely regulated in the peripubertal PVN and that these proteins may be playing a broader or different role in 5-HT\textsubscript{1A} receptor-mediated signaling and desensitization in the peripubertal PVN than previously established in the adult PVN. The data also demonstrate that 5-HT\textsubscript{1A} receptor-mediated pathways may be differentially responsive to different classes of 5-HT\textsubscript{1A} agonists. (+)8-OH-DPAT (an aminotetralin), acted as a “full” agonist on the 5-HT\textsubscript{1A} receptor-mediated neuroendocrine and kinase responses. In contrast, tandospirone (an azapirone) exhibited “partial” agonist activity with regards to Akt activation despite “full” agonist activity in stimulating increases in both oxytocin and ACTH and in the activation of ERK. 5-HT\textsubscript{1A} receptor-mediated activation of ERK in the peripubertal PVN is a rapid and prolonged response, unlike the rapid but transient nature of this response observed in the adult PVN. These studies also
suggest that ERK activation may be expressed only in certain populations of neuroendocrine cells in the peripubertal PVN. We have also determined that 5-HT_{1A} receptor-mediated increases in the phosphorylation of GSK3β were attenuated in fluoxetine-treated peripubertal rats in the absence of changes in Akt activation. These data suggest that fluoxetine may be altering phosphatase activity or that Akt may not be the kinase responsible for GSK3β phosphorylation in PVN or at this developmental age.

These unique aspects of 5-HT_{1A} receptor signaling in the peripubertal brain may be involved with differences in therapeutic and/or side effects of SSRIs in adult vs. young patients or may represent novel drug targets to treat mood disorders in children and adolescents.

5-HT_{1A} receptor signaling in the peripubertal PVN

In the peripubertal PVN, activation of 5-HT_{1A} receptors leads to increases in plasma levels of both oxytocin and ACTH hormones and increases the levels of activated (phosphorylated) ERK (pERK) in the PVN (Chapter 3). Furthermore, we determined that 5-HT_{1A} receptor activation increases activated (phosphorylated) Akt (pAkt) in the peripubertal PVN (Chapter 6).

5-HT_{1A} receptors activate the mitogen activated protein kinase (MAPK) ERK in the peripubertal PVN. In the adult PVN, activation of ERK is a rapid but transient response, as the aminotetralin “full” agonist (+)8-OH-DPAT significantly elevates levels of activated ERK at 5 minutes which returns to basal levels by 15
minutes post-injection (Crane et al., 2007). Tandospirone, an azapirone “partial” agonist at postsynaptic 5-HT$_{1A}$ receptors, also increases pERK in the adult rat PVN (Sullivan et al., 2005) although the time- and dose-dependence of tandospirone on 5-HT$_{1A}$ receptor-mediated pERK responses has not been determined in the adult PVN. In the peripubertal PVN, (+)-8-OH-DPAT and tandospirone also produced rapid (≤ 5 minutes) increases in pERK. However, in contrast to adult PVN, 5-HT$_{1A}$ receptor-mediated activation of ERK in the peripubertal PVN was prolonged, remaining elevated over basal at least 30 minutes after injection. These data demonstrate that (+)-8-OH-DPAT produced a more prolonged 5-HT$_{1A}$ receptor-mediated increase in pERK in the PVN of peripubertal rats as compared to that previously published in adult PVN. Age-dependent effects on ERK signaling have also been demonstrated in response to peptide-induced activation of NMDA receptors in the spinal cord of rats (Chiu et al., 2010). Prolonged ERK activation in the PVN of peripubertal rats could impact a variety of cellular functions under the control of ERK. In a variety of mammalian cell lines, the duration of ERK signaling is associated with distinct cell behavior (Traverse et al., 1992; Nguyen et al., 1993; Mansour et al., 1994; Sharp et al., 1997; Whalen et al., 1997; Murphy et al., 2002; Murphy et al., 2004; Sinnett-Smith et al., 2004). For example, prolonged but not transient ERK activation was associated with (1) neuronal differentiation in PC-12 cells (Traverse et al., 1992), (2) nuclear translocation of ERK in PC12 cells (Nguyen et
al., 1993), and (3) c-fos accumulation and DNA synthesis in Swiss 3T3 cells (Sinnett-Smith et al., 2004). In consideration of our previous findings that ERK is required for SSRI-induced desensitization of 5-HT_{1A} receptor-mediated neuroendocrine responses in the adult PVN, a more prolonged ERK signaling in the immature brain could potentially induce a more rapid desensitization of 5-HT_{1A} receptor-mediated hormone responses upon administration of SSRIs.

In Chapter 5, we demonstrated that cells in the peripubertal PVN that were immunoreactive for oxytocin were localized distinct from cells displaying immunoreactivity for 5-HT_{1A} receptor-mediated pERK. Our preliminary data suggested that these markers were localized in distinct cell populations in the adult PVN as well. These data are consistent with the literature suggesting that little to no receptor-stimulated ERK is observed in oxytocin-containing cells (Wang et al., 2006; Blume et al., 2009; Jankord et al., 2010). In the adult PVN, intact ERK signaling is necessary for SSRI-induced desensitization of 5-HT_{1A} receptor-mediated oxytocin neuroendocrine responses (Jia et al., 2007). The findings of the current study suggest that the effects of ERK on SSRI-induced desensitization of 5-HT_{1A} receptor-mediated oxytocin responses in the adult PVN may be induced via a cell-to-cell mechanism rather than via a more direct intracellular communication between signaling pathways. Nevertheless, the present immunohistochemical studies indicate that 5-HT_{1A} receptors mediating oxytocin responses exhibit a different localization than those 5-HT_{1A} receptors
responsible for the activation of ERK. It is currently unknown if ERK is necessary for SSRI-induced desensitization of 5-HT$_{1A}$ receptor-mediated oxytocin responses in the peripubertal PVN. If so, the current data suggest that similar cell-to-cell mechanisms are likely operative in the immature brain. In contrast, receptor-stimulated pERK localizes with corticotrophin releasing hormone (CRH)-immunoreactive cells in the PVN (Khan et al., 2007). Consistent with these findings, we have preliminary data that suggest, in the peripubertal PVN, 5-HT$_{1A}$ receptor-mediated pERK immunoreactivity localized with cells immunoreactive for CRH. Since CRH-containing cells can project to other areas of the PVN (Sakanaka et al., 1987), and that 5-HT$_{1A}$ receptor-mediated activation may occur in CRH containing cells, it is possible that CRH-containing could exert a wide range of control over multiple cell subtypes within the PVN and in other areas of the hypothalamus.

5-HT$_{1A}$ receptors activate protein kinase B (Akt) in the peripubertal PVN. Due to recent evidence linking the PI3K/Akt/GSK3 pathway to the pathology of psychological disorders including chronic stress, depression, and schizophrenia (Beaulieu et al., 2009) and actions of psychoactive drugs (Beaulieu et al., 2004; Li et al., 2004), 5-HT$_{1A}$ receptor signaling of Akt was investigated in the peripubertal PVN (Chapter 6). These studies are among the first to identify that activation of a G protein coupled receptor results in the phosphorylation of Akt in the hypothalamus in vivo. The PI3K/Akt pathway had been classically
characterized as being activated via growth factor receptors such as tyrosine kinase receptors. However, recent studies show that GPCRs (e.g. D2 dopamine and 5-HT1A receptors) also can activate this pathway (Beaulieu et al., 2005; Adayev et al., 1999; Cowen et al., 2005; Hsiung et al., 2005). For full activation, Akt must be phosphorylated at two residues, Thr308 and Ser473. Each of these sites is phosphorylated by different upstream kinases: PDK1 phosphorylates Thr308 (Alessi et al., 1997; Stephens et al., 1998) and mTOR has been identified as the kinase for Ser473 (Sarbassov et al., 2005). Phosphorylation at Ser473 by mTOR appears to occur in the cytoplasm and subsequently assists in the recruitment of Akt to the plasma membrane and so that it can be phosphorylated at Thr308 by PDK1 (Andjelkovic et al., 1997; Scheid and Woodgett, 2003).

Activation of 5-HT1A receptors in vivo resulted in increases in phosphorylated Akt in the peripubertal PVN. In the current studies, injection with two different structural classes of 5-HT1A receptor agonists, (+)8-OH-DPAT and tandospirone, resulted in a rapid phosphorylation of Akt at both Thr308 and Ser473, although (+)8-OH-DPAT appeared to induce a higher magnitude of phosphorylation at both Akt phosphorylation sites. Considering that Akt may be first phosphorylated at Ser473 by mTOR, it is conceivable that (+)8-OH-DPAT (at 0.20 mg/kg) may induce more effective coupling of 5-HT1A receptors to the activation of mTOR than tandospirone. Alternatively, (+)8-OH-DPAT may be more efficacious than tandospirone in activating 5-HT1A receptors responsible for the
phosphorylation of Akt. This will be discussed further in another section of this chapter.

5-HT\textsubscript{1A} receptor-mediated activation of Akt in peripubertal vs. adult PVN was examined in a separate study (data not shown). Both adult and peripubertal (PD 42) rats were given a challenge injection of 0.20 mg/kg (+)8-OH-DPAT and sacrificed 10 minutes later. 5-HT\textsubscript{1A} receptor-mediated phosphorylation of Akt was significantly less in the adult PVN than peripubertal PVN at both Thr308 (75% over basal in adult PVN compared to 100% over basal in peripubertal PVN) and Ser473 sites (40% over basal in adult PVN compared to 79% over basal in peripubertal PVN) (Petrunich-Rutherford et al., unpublished data). 5-HT\textsubscript{1A} receptors in the adult PVN could potentially be less effective in activating the PI3K/Akt pathway than in young rats. However, considering that 5-HT\textsubscript{1A} receptor-mediated increases in pERK are rapid and transient in the adult PVN (Crane et al., 2007), it is more likely that 5-HT\textsubscript{1A} receptor-mediated increases in pAkt may display a more transient time course of activation in the adult PVN than that observed in peripubertal PVN. In either case, a more prolonged 5-HT\textsubscript{1A} receptor-mediated activation of Akt in the peripubertal PVN could potentially impact a wide variety of cellular functions.

Together, these data provide the first \textit{in vivo} demonstration that 5-HT\textsubscript{1A} receptors can activate Akt in the peripubertal PVN. Given the previous studies demonstrating that 5-HT\textsubscript{1A} receptors can stimulate hormone responses and
activate ERK in the peripubertal PVN (Chapter 3), these findings are the first to show that 5-HT\textsubscript{1A} receptors can mediate the concomitant activation of multiple downstream kinase effectors in peripubertal hypothalamic PVN.

5-HT\textsubscript{1A} receptor-mediated neuroendocrine responses and activation of ERK in the peripubertal PVN: independent pathways? In the adult rat hypothalamic PVN, 5-HT\textsubscript{1A} receptors mediate neuroendocrine and ERK kinase responses via separate and independent pathways mediated by separate subsets of G\textsubscript{α} proteins. The data from the current studies suggest that 5-HT\textsubscript{1A} receptor-mediated signaling of neuroendocrine responses and ERK activation may also be independent in the peripubertal PVN, although the role of G\textsubscript{α} proteins in these signaling pathways were harder to discern. As previously mentioned, the immunohistochemical study indicated that 5-HT\textsubscript{1A} receptor-mediated activation of ERK does not occur on oxytocin-containing cells in the peripubertal PVN, indicating that increases in plasma levels of oxytocin and increases in PVN levels of pERK are activated in separate cell groups and therefore via independent pathways (Chapter 5). Furthermore, it seems unlikely that 5-HT\textsubscript{1A} receptor-mediated pERK contributes to the increases observed in plasma concentrations of oxytocin and ACTH, considering that 0.10 mg/kg (+)8-OH-DPAT significantly increased plasma levels of oxytocin and ACTH but did not significantly increase pERK levels in the peripubertal PVN (Chapter 3). However, these studies did not directly determine the independence of 5-HT\textsubscript{1A} receptor-mediated pathways and merely
suggest a possible independence of 5-HT$_{1A}$ receptor-mediated neuroendocrine responses and activation of ERK in the peripubertal PVN. The specific roles of $G\alpha$ proteins in 5-HT$_{1A}$ receptor-mediated signaling in the peripubertal PVN were also investigated; these findings are discussed further in another section of this chapter.

In the literature, it is not clear whether or not Akt and ERK protein kinase pathways are activated as a result of one shared pathway or multiple independent pathways. In a transfected Chinese hamster ovary (CHO) cell line, both the 5-HT$_{1A}$ receptor-mediated activation of ERK and Akt were reported to be pertussis toxin-sensitive and PI3K-dependent (Hsiung et al., 2005). However, the 5-HT$_{1A}$ receptor-mediated activation of Akt, but not 5-HT$_{1A}$ receptor-mediated ERK, is PI3K-dependent in the hippocampal neuron-derived cell line HN2-5 (Adayev et al., 1999). At this point, it appears that the dependence/independence of ERK and Akt phosphorylation depends on the cell type. The current studies indicated that ERK and Akt phosphorylation follow a similar time course (i.e. elevated within 5 minutes of agonist injection, remained elevated until around 15 minutes post-injection, and slowly returned to basal levels). Therefore, we are unable to say with certainty if ERK activation leads to Akt phosphorylation or vice versa, or if these pathways are independently stimulated in the peripubertal PVN.
“Full” vs. “partial” agonism at 5-HT\textsubscript{1A} receptor-mediated responses in the peripubertal PVN.

In the literature, (+)8-OH-DPAT is considered a “full” agonist and tandospirone is considered a “partial” agonist at post-synaptic 5-HT\textsubscript{1A} receptors. However, most studies have examined the 5-HT\textsubscript{1A} receptor mediated-responses induced by these agonists in cell cultures or in brain regions other than the hypothalamus. The studies from Chapter 3 indicated that both (+)8-OH-DPAT and tandospirone had comparable maximal efficacies in stimulating oxytocin, with (+)8-OH-DPAT being a more potent agonist than tandospirone. The maximal efficacies of (+)8-OH-DPAT and tandospirone on ACTH responses were also comparable, and maximally effective doses of the aminotetralin or azapirone agonists appear to produce comparable increases in pERK in PVN. Therefore, both (+)8-OH-DPAT and tandospirone act as “full” agonists on 5-HT\textsubscript{1A} receptor-mediated neuroendocrine responses and ERK activation in the peripubertal PVN. However, in Chapter 6, it appeared that the maximal increases of pAkt at both Thr308 and Ser473 induced by (+)8-OH-DPAT are greater than that induced by tandospirone. Therefore, with respect to Akt activation, (+)8-OH-DPAT may be considered a “full” agonist and tandospirone a “partial” agonist in contrast with the data demonstrating that both drugs are “full” agonists on 5-HT\textsubscript{1A} receptor-mediated neuroendocrine and ERK responses (as reported in Chapter 3). These data demonstrated that the classification of
agonists as “full” or “partial” are dependent on the respective receptor-mediated response, despite the fact that multiple responses can be stimulated by the same receptor in the same brain region.

**Effects of chronic fluoxetine on 5-HT\textsubscript{1A} receptor signaling in the peripubertal PVN**

In the adult rat PVN, 5-HT\textsubscript{1A} receptor-mediated neuroendocrine and kinase pathways are differentially regulated by selective serotonin reuptake inhibitors (SSRIs). Chronic treatment with SSRIs decreases levels of G\textsubscript{az} proteins and desensitizes 5-HT\textsubscript{1A} receptor-mediated oxytocin and ACTH hormone responses (Raap et al., 1999) but has no effect on levels of G\textsubscript{ai/o} proteins and does not desensitize the 5-HT\textsubscript{1A} receptor-mediated increase in pERK in the PVN (Jia et al., 2006). Furthermore, intact ERK signaling is necessary for SSRI-induced desensitization of 5-HT\textsubscript{1A} receptor-mediated neuroendocrine responses, since pharmacological blockade of MAPK kinase (the activating kinase for ERK) during SSRI treatment abolishes both the SSRI-induced desensitization of 5-HT\textsubscript{1A} receptor signaling of hormone responses and reductions in associated G\textsubscript{az} proteins (Jia et al., 2007). In the peripubertal brain, chronic fluoxetine also desensitizes 5-HT\textsubscript{1A} receptor-mediated oxytocin and ACTH responses (Chen et al., 2006); however, neither 7 nor 14 days of fluoxetine treatment had any effect on either membrane- or cytosol-associated G\textsubscript{az} protein levels in the peripubertal hypothalamus (Chapter 4). G\textsubscript{az} is the exclusive G\textsubscript{a} protein mediator of increases
in plasma hormone concentrations stimulated by 5-HT$_{1A}$ receptor activation in the adult and the only G$\alpha$ protein reduced by SSRIs in the adult brain (Serres et al., 2000a; Jia et al., 2006). Therefore, the data from the current fluoxetine study described in Chapter 4 suggest that either 5-HT$_{1A}$ receptor-mediated neuroendocrine responses are mediated via a different subset of G$\alpha$ protein(s) in the peripuberal PVN or that SSRIs desensitize 5-HT$_{1A}$ receptor-mediated neuroendocrine responses in the peripuberal PVN via a different mechanism—one not dependent on the downregulation or reductions in the levels of G$\alpha$ proteins. Differences in the roles and regulation of G$\alpha$ proteins will be discussed further in the section on the unique regulation of G$\alpha$ proteins in the peripuberal brain.

We also determined that chronic fluoxetine (7 and 14 days) had differential effects on the ERK and Akt kinase pathways. 5-HT$_{1A}$ receptor-mediated phosphorylation of ERK and phosphorylation of Akt is generally not affected with either 7 or 14 days of fluoxetine. However, 14 days of injections with either saline or fluoxetine rendered the animals hyporesponsive to the effects of (+)8-OH-DPAT on 5-HT$_{1A}$ receptor-mediated activation of ERK (Chapter 4) and on 5-HT$_{1A}$ receptor-mediated phosphorylation of Akt (Chapter 6). Given the blunted response in chronic saline-treated rats, there may be stress-related effects due to the additional 7 days of injection in the peripuberal period. Alternatively, starting injections at PD 28 (as with the 14 day treatment paradigm used) as
opposed to PD 35 (as with the 7 day treatment paradigm used) may have exposed the animals to injection stress at a critical point in postnatal development. Although we did not observe any specific fluoxetine-induced effects at the level of Akt, its downstream target GSK3β was differentially affected by the 7 and 14 day chronic treatments. There was no hyporesponsive effect of 14 days of injection (as observed with (+)8-OH-DPAT challenge on pERK and pAkt) in either saline or fluoxetine-treated animals on phosphorylated levels of GSK3β. We did observe almost a complete desensitization of (+)8-OH-DPAT-induced increases in pGSK3β with 7 days fluoxetine treatment. With 14 days of fluoxetine treatment, this desensitization is not as marked, suggesting some neuroadaptive response to fluoxetine in the developing peripubertal brain. Alternatively, starting treatment earlier in postnatal development may not be having a robust effect on (+)8-OH-DPAT-stimulated pGSK3β, or fluoxetine could possibly be modulating the protein levels or activity of a pGSK3β-specific phosphatase, which may account for the differential effects on GSK3β but not Akt phosphorylation.

**Unique regulation of Gα proteins in the peripubertal PVN.**

*Developmental differences in the regulation of 5-HT$_{1A}$ receptor-associated Gα proteins in the PVN.* With regards to protein levels of 5-HT$_{1A}$ receptor-associated Gα proteins, we determined that Gao proteins, but none of the other Gα protein subtypes tested, exhibit age-dependent increases. Gai1, Gai2, Gai3, and Gaz
proteins (but not Gαo proteins) are present in the PVN, hippocampus, and frontal cortex at their adult levels in both prepubertal (PD 28) and peripubertal (PD 42) rats, whereas Gαo proteins steadily increase with age. This is consistent with a previously published study that determined that levels of Gα proteins change throughout several brain regions during early postnatal development. Gαi1, Gαi2, and Gα3 proteins reach adult levels around postnatal day 18 in the cortex, thalamus, and hippocampus, whereas Gαo proteins continue to increase past early postnatal development in these regions (Ihnatovych et al., 2002). Together, these data indicate that, besides Gαo, all other Gα proteins with the capacity to couple to 5-HT1A receptors are present in the peripubertal PVN at levels similar to that in the adult PVN. The developmental increase in Gαo proteins may impact 5-HT1A receptor signaling if the lower density of Gαo proteins in peripubertal vs. adult PVN alters the effective coupling of the 5-HT1A receptor to the respective signaling pathways in the brain of younger vs. adult rats. For example, if the relative abundance of other proteins (like Gαi3 and Gαz) makes it less likely for the 5-HT1A receptor to couple to Gαo, then Gαo proteins may be precluded from signaling in a manner similar to that in the adult brain. Unfortunately, the limitation of western blotting is that comparisons cannot be made between the relative levels of Gα proteins to one another. The results from other experiments, however, indicate that there may be some other difference in
the Gα proteins (and not just the physical availability) that may explain differences in 5-HT1A receptor signaling in the peripubertal vs. adult PVN.

In Chapters 4 and 6, we intended to thoroughly investigate the specific roles of Gα proteins in 5-HT1A receptor signaling in the peripubertal PVN. However, we discovered some key differences in the regulation of Gα proteins that prevented the determination of the specific roles that these Gα proteins play in 5-HT1A receptor-mediated signaling in the peripubertal PVN. These differences in regulation are described in the next few paragraphs, and the conclusions that we made regarding the specific roles of Gα proteins in 5-HT1A receptor-mediated signaling pathways are discussed in the next section in this chapter.

Pertussis toxin (PTX) is a bacterial toxin that catalyzes the addition of an ADP-ribose group to a c-terminal cysteine residue on Gai1, Gai2, Gai3, and Gao proteins, which functionally inactivates these respective proteins. PTX-insensitive Gαz proteins lack this critical cysteine residue necessary for PTX-induced ADP ribosylation. In the adult rat in vivo, an intra-PVN injection with PTX induces a significant decrease (~50%) in the amount of each of the PTX-sensitive Gα proteins in the hypothalamus at 72 hours post-injection (Garcia et al., 2006). This reduction is not due to an alteration in the antibody recognition of the ADP-ribosylated Gα proteins, since in vitro incubation of adult hypothalamic membranes with PTX has no effect on the levels of Gα proteins measured with western blotting (Petrunich-Rutherford et al., unpublished data).
In contrast to the effects of PTX in adults, in peripubertal rats, an intra-PVN injection of PTX did not reduce all subsets of Ga\textsubscript{i/o} proteins in the hypothalamus (as in the adult) but only reduced Ga\textsubscript{i1} and Ga\textsubscript{i2}. Ga\textsubscript{i3} protein levels in the hypothalamus remained unchanged and Ga\textsubscript{o} protein levels in the hypothalamus increased two-fold. It is important to note that there may have been changes in specific Ga proteins at the level at the PVN that were masked by changes in the whole hypothalamus. However, considering the same Ga proteins were measured in the whole hypothalamus with a comparable incubation time of drug (72 hours) in this study as the one previously published in adults (Garcia et al., 2006), these data indicate that there may be a developmental difference in the PTX-sensitivity of Ga proteins in the hypothalamus. Although unlikely, a difference in the Ga\textsubscript{i3}/Ga\textsubscript{o} protein structure may be preventing ADP ribosylation in the peripubertal rat brain. It is more likely that there is a difference in post-translational modifications of the proteins that may be preventing ADP ribosylation and subsequent reduction in membrane-associated levels of these proteins. There is also a possibility that a 72-hour incubation time is not sufficient (or is perhaps too short) to functionally inactivate Ga\textsubscript{i3} and Ga\textsubscript{o} proteins. It could be that Ga\textsubscript{i3} and Ga\textsubscript{o} proteins are more resilient to ADP-ribosylation and begin to recover from the effect of the toxin or are replenished by transcription and translation of their respective genes before the end of the three-day time period. Indeed, the increased levels of Ga\textsubscript{o}
may be due to a robust compensatory increase to the initial effects of PTX. There
has been one other study comparing the effects of PTX in peripubertal vs. adult
brain: using adult rat brain membranes, GABA_B receptor/G protein binding was
reduced upon incubation with PTX in cortex, hippocampus, and cerebellum but
not the striatum. However, when PTX was incubated with peripubertal rat brain
membranes, GABA_B receptor/G protein binding was reduced in striatum and
hippocampus but not cortex or cerebellum (Knott et al., 1993). In either case, in
vivo or in vitro, it seems as if certain Gα proteins (namely Gαi3 and Gαo) have not
functionally matured in the peripubertal brain and may possibly impact the
signaling of the 5-HT_1A receptor in the PVN and other regions in the brain.

We also observed developmental differences in the PVN upon chronic
treatment with fluoxetine in the peripubertal rat. Chronic fluoxetine reduces
levels of Gαz proteins in the PVN of adult rats. Our preliminary studies
indicated that, in the peripubertal PVN, chronic fluoxetine did not reduce Gαz
proteins but did reduce Gαi3 and Gαo (Chen et al., 2006). However, upon
replicating the treatment paradigm for both 7 and 14 days, we determined that
chronic fluoxetine did not reduce Gαz proteins but also did not reduce any of the
other Gα proteins (cytosol-associated or membrane-associated) in the
peripubertal PVN (Chapter 4). Therefore, SSRIs may be desensitizing 5-HT_1A
receptor-mediated neuroendocrine responses in the peripubertal PVN via a
different mechanism as opposed to reducing levels of Gαz proteins in the
hypothalamus, as in the adult. For example, SSRI treatment in peripubertal rats may be affecting the function of regulators of G protein signaling (RGS) proteins. The RGSZ protein functions to deactivate Gαz subunits and is present in the hypothalamus. Members of the RGSZ protein family have been shown to influence mu receptor signaling in the central nervous system by sequestering Ga proteins (Garzon et al., 2005). SSRIs may be causing RGSZ proteins to inactivate or sequester, but not cause a reduction in the levels of Gαz proteins in the peripubertal hypothalamus. Alternatively, chronic fluoxetine may be altering the balance of Ga proteins in lipid rafts, and subsequently the signaling activity of these proteins, as has been observed with chronic escitalopram (an SSRI) on Gαs proteins in vitro (Zhang and Rasenick, 2010).

Roles of 5-HT1A receptor-associated Gα proteins in the PVN. Intra-PVN injection with PTX caused a reduction of hypothalamic levels of Gαi1 and Gαi2 proteins and increased Gαo proteins (Chapter 4, figure 13). We found no significant reductions in 5-HT1A receptor-mediated neuroendocrine responses; therefore, this data suggests that Gαi3, Gαo, and/or Gαz could potentially mediate 5-HT1A receptor stimulation of hormone responses in the PVN of peripubertal rats. It is likely that Gαi3, Gαo, and/or Gαz proteins may mediate 5-HT1A receptor activation of ERK considering that there were also no significant reductions of 5-HT1A receptor-mediated phosphorylation of ERK in the PVN. However, the PTX-induced increases in Gαo may be responsible for the elevations observed in
basal levels of activated ERK in the PVN. Similarly, we observed slightly elevated basal levels of phosphorylated Akt at Ser473, although this increase did not reach statistical significance. This increase in basal levels of pAkt may have precluded any further increase in pAkt at Ser473 by 5-HT$_{1A}$ receptor activation. Therefore, 5-HT$_{1A}$ receptors may be signaling via G$_{ai1}$ or G$_{ai2}$ proteins to mediate Akt activation, although we cannot completely rule out any potential contribution of G$_{ai3}$, G$_{ao}$, or G$_{az}$ in mediating the phosphorylation and activation of Akt in the peripubertal PVN.

To determine the specific G$_{a}$ protein contribution to 5-HT$_{1A}$ receptor-mediated signaling pathways in the peripubertal PVN, a more selective method is necessary, like adenoviral-delivered siRNA for G$_{ai3}$ and G$_{ao}$ proteins. This will be discussed further in the last section of this chapter.

Alternate signaling mediators of 5-HT$_{1A}$ receptor-mediated responses in peripubertal PVN. Although the studies in this dissertation have focused on the contributions of G$_{a}$ proteins to 5-HT$_{1A}$ receptor-mediated signaling in the peripubertal PVN, many studies have revealed that GPCRs can mediate the signaling of ERK and Akt via G$_{b\gamma}$ subunits or even via β-arrestin. G$_{b\gamma}$ subunits have been shown to activate ERK (Goubaeva et al., 2003; Mukhin et al., 2000) and Akt (Konishi et al., 1995; Guzmán-Hernández et al., 2009); therefore, G$_{b\gamma}$ could potentially mediate 5-HT$_{1A}$ receptor-mediated responses rather than G$_{a}$ proteins in the peripubertal PVN. Although β-arrestin has been classically involved with receptor
desensitization, uptake, and degradation, in certain situations it can serve as a signaling mediator for GPCR/G protein-independent signaling. For example, β-arrestin has been shown to activate ERK in response to activation by (1) β2 adrenergic receptors (Shenoy et al., 2006), (2) GPR54 receptors (Pampillo et al., 2009), and (3) angiotensin II receptors (Ahn et al., 2004). Either Gβγ subunits or β-arrestin could be potential second messengers for Ga protein-independent signaling of the respective 5-HT1A receptor-mediated responses in the peripubertal PVN.

It is also possible that 5-HT1A receptor-mediated activation of protein kinase pathways could be due to transactivation of growth factor receptors by the 5-HT1A receptor in the PVN. One potential growth factor receptor is insulin-like growth factor 1 receptor (IGF-1R). IGF-1R has been localized to the PVN (Saeed et al., 2007) and can be transactivated by another GPCR, the GABA_B receptor. Transactivation of IGF-1R by GABA_B results in increased levels of phosphorylated Akt in primary cerebellar granule neurons (Tu et al., 2010). Although the interaction of 5-HT1A receptor with IGF-1R pathways has not been examined in the hypothalamus in vivo, 5-HT1A receptors have been shown to regulate IGF-1R mRNA and protein levels in the spinal cord (Bonnefont et al., 2007).
Clinical significance: some implications for therapeutic strategies for children and adolescents.

Differences observed in hypothalamic 5-HT$_{1A}$ receptor signaling and the properties of Go proteins and their signaling may only make up a fraction of developmental differences in brain function between a developing animal and an adult animal. For example, there are differences observed between the peripubertal and adult groups in: (1) cocaine-induced behavior (Laviola et al., 1995; Schramm-Sapyta et al., 2004); (2) nicotine-induced sensitization of cocaine responses (Collins and Izenwasser 2004); (3) motor activity and cortex activation in response to an NMDA receptor modulator (Pesic et al., 2010); and (4) receptor regulation induced by treatment with a tricyclic antidepressant (Deupree et al., 2007). One study observed that there was no overall differences in the magnitude of behavior induced by bupropion (an antidepressant used in treating nicotine addiction) in the peripubertal and adult animals, but that the developing animals may be more sensitive to lower doses of the drug. Furthermore, in neuroendocrine neurons, a balance between GABA and glutamate (NMDA and AMPA) receptor subtypes controls intracellular levels of calcium. One group has observed a developmental shift in the levels of AMPA receptors controlling this as the animal matures (Constantin et al., 2010). These studies along with the studies described in this dissertation have serious implications for the use and abuse of drugs by children and adolescents. Even a developmental difference
that seems inconsequential (such as a more prolonged activation of ERK) may have far-reaching consequences, such as with the effectiveness of antidepressant drugs used in children and adolescents, the side effect profile of psychotropic drugs in younger populations, and the permanence of brain changes upon maturation. Some observations of young animals treated with a variety of drugs reveal later alterations of adult behavior and brain function: (1) ethanol treatment alters locomotor behavior as an adult (Roskam and Koch, 2009) and (2) nicotine increases anxiety behavior and alters CRH and neuropeptide Y systems (Slawecki et al., 2005).

**Future directions for the present studies**

There are several courses of action that could be pursued to further clarify the mechanisms of 5-HT$_{1A}$ receptor-mediated signaling and desensitization in the peripubertal PVN.

First of all, a few of the pharmacological tools employed in these studies (namely PTX and fluoxetine) did not produce the anticipated effects on G$\alpha$ proteins. To completely clarify the role of specific G$\alpha$ proteins in 5-HT$_{1A}$ receptor-mediated neuroendocrine responses and kinase activation, a more specific tool such as adenoviral-delivered siRNA or antisense oligonucleotides for the specific G$\alpha$ proteins must be utilized. This will also assist in determining if 5-HT$_{1A}$ receptor-mediated pathways in the peripubertal PVN are independent and, if so, if these pathways are mediated by separate subsets of G$\alpha$ proteins.
It may also be important to determine if ERK or Akt protein kinase pathways contribute to the respective 5-HT\textsubscript{1A} receptor-mediated responses. In a separate experiment, we gave peripubertal rats an intra-PVN injection of the PI3K inhibitor LY294002 to assess the role of Akt’s upstream kinase PI3K in 5-HT\textsubscript{1A} receptor-mediated signaling of neuroendocrine, ERK, and Akt responses in the peripubertal PVN. Although 5-HT\textsubscript{1A} receptor-mediated oxytocin and ACTH responses, but not pERK responses, were partially reduced, there were no significant effects of the PI3K inhibitor on the phosphorylation of Akt at either Thr308 or Ser473 (Petrunich-Rutherford et al., unpublished data). Therefore, it may be possible that PI3K may contribute to 5-HT\textsubscript{1A} receptor-mediated neuroendocrine responses in the peripubertal PVN. An alternative explanation for the lack of PI3K inhibition on 5-HT\textsubscript{1A} receptor-mediated ERK and Akt kinase responses could be that 5-HT\textsubscript{1A} receptors may be activating ERK and Akt via a PI3K-independent mechanism, which has been observed in other systems (Alessi et al., 1996; Sable et al., 1997; Yano et al., 1998).

Further research is necessary to determine specifically which cells contain 5-HT\textsubscript{1A} receptor-mediated pERK in peripubertal PVN. Currently, a protocol for immunohistochemical double labeling of CRH and 5-HT\textsubscript{1A} receptor-stimulated pERK is in progress to optimize colocalization of these markers in both peripubertal and adult PVN using perfused rather than post-fixed brain tissue. Thus far, it appears that some of the factors involved with perfusion (anesthesia,
stress, perfusion itself) increases basal levels of ERK such that further stimulation of 5-HT$_{1A}$ receptors by (+)8-OH-DPAT can not be clearly discerned. More studies are necessary to determine the impact of these factors on ERK stimulation in the PVN.

As mentioned previously, in the adult PVN (Petrunich-Rutherford et al., unpublished data), 5-HT$_{1A}$ receptors increase the phosphorylation of Akt; however, the full time course and dose-responsiveness has not been investigated in this age group with either (+)8-OH-DPAT or tandospirone. Also, it is unknown at this time whether tandospirone acts as a “full” or “partial” agonist at 5-HT$_{1A}$ receptor-mediated oxytocin, ACTH, pERK, and pAkt responses in the adult PVN. It would be interesting to determine if the actions of tandospirone in the adult PVN are different compared with the “full” agonist actions on neuroendocrine responses and pERK yet “partial” agonist on pAkt responses in the peripubertal PVN.

Another key piece of information would be to determine if intact ERK or Akt signaling pathways are necessary for SSRI-induced desensitization of 5-HT$_{1A}$ receptor-mediated neuroendocrine responses in the peripubertal PVN. Furthermore, considering that 5-HT$_{1A}$ receptor-mediated phosphorylation of GSK3β (but not 5-HT$_{1A}$ receptor-mediated activation of its upstream kinase Akt) was desensitized following chronic fluoxetine, another potential area for study is the regulation of phosphatases responsible for dephosphorylating ERK and Akt.
upon chronic antidepressant or acute 5-HT_{1A} receptor stimulation. Phosphatases have been implicated in stress and the effects of chronic antidepressants in other models. In the hippocampus, chronic stress increases hippocampal levels of a phosphatase specific for MAPK, and antidepressants normalize the levels of this phosphatase (Duric et al., 2010). The protein level of PP1 phosphatase (the primary phosphatase for GSK3β in COS-7 cell cultures, Hernandez et al., 2010), was increased in the adult frontal cortex and hippocampus in a rat model of depression (Feng et al., 2003). Therefore, pursuing the regulation of phosphatases in the peripubertal and adult PVN may represent a new and exciting research area.

In conclusion, although several facets of 5-HT_{1A} receptor-mediated signaling in the peripubertal PVN have been determined, it seems that even more questions have been generated about the signaling and regulation of this receptor in the immature brain, and much more research is needed to fully elucidate the role of this receptor in mood disorders and the effects of antidepressants.
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VITA

Maureen Lynn Petrunich Rutherford was born April 14, 1983, in Hammond, Indiana and is the daughter of Michael Petrunich and Mary Kay Ader and stepfather Patrick Ader. In 2001, she graduated as valedictorian from George Rogers Clark High School. She then attended Indiana University Northwest in Gary, Indiana, where she earned Bachelor of Science degrees in Chemistry and Psychology in May 2005. During the course of her undergraduate studies, she carried out research projects in both chemistry and psychology under the guidance of Drs. Attila Tuncay and Mark Hoyert, respectively. Maureen’s interest in the neurochemical and biological basis of psychological disorders and the treatment of these disorders led her to pursue a graduate degree in the Neurosciences.

Maureen entered the Neuroscience Graduate Program at Loyola University Chicago in August of 2005. She joined the laboratory of Dr. George Battaglia in 2006, where she began her work on serotonin 1A receptor signaling in the peripubertal hypothalamic paraventricular nucleus. During her time at Loyola, Maureen has presented her research at the Society for Neuroscience conferences in San Diego and Washington DC, as well as at local Chicago Chapter meetings. She has served as treasurer and secretary for the LUMC Graduate Student
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After completing her Ph.D., Maureen plans to pursue a career in teaching. One of her goals is to establish a program in Neuroscience at an undergraduate university in northwest Indiana that would foster research opportunities for undergraduate students. Maureen married her husband Billy Rutherford in 2009. They currently reside in Portage, Indiana.