Ovarian Steroid Regulation of Serotonin 5-HT_{1A} Receptor and Reuptake Transporter at the Protein and Functional Levels in Female Macaques

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CERTIFICATE OF APPROVAL

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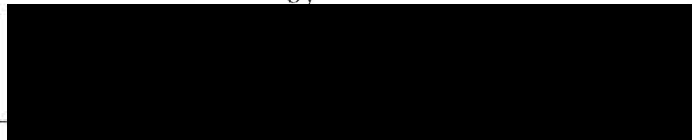
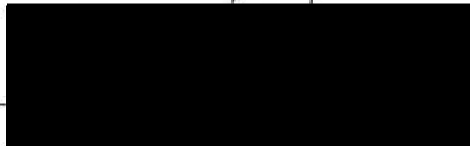
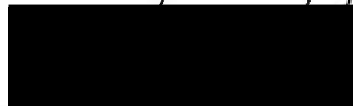


Cynthia Bethea, Ph.D.





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ABSTRACT

The serotonin 5-HT_{1A} receptor and reuptake transporter (SERT) play important roles in serotonin neurotransmission and mental health. Estradiol (E) and progesterone (P) decrease 5-HT_{1A} autoreceptor and SERT mRNA levels in the macaque dorsal raphe nucleus (DRN). In this study, I asked whether E and P regulate 5-HT_{1A} binding and function, G_{α} subunit protein expression, SERT binding sites, SERT protein expression, and function. Quantitative autoradiography for 5-HT_{1A} receptors, G proteins, and SERT proteins using [^{3}H]8-OH-DPAT, [^{35}S]GTP-γ-S, and [^{3}H]citalopram, respectively, was performed on brain sections of rhesus macaques from four treatment groups: ovariectomized controls (OVX), E (28 d), P (28 d), and E (28 d) plus P (the last 14 d) treated. Western blot analyses for G_{α} subunits and SERT were performed on raphe extracts from cynomolgus macaques that were OVX or OVX treated with equine estrogens (EE, 30 months). [^{3}H]Serotonin uptake was measured in forebrain synaptosomal preparations of the same rhesus macaques used for autoradiography studies. In the 5-HT_{1A} study, postsynaptic 5-HT_{1A} binding sites in the hypothalamus were decreased by E or E + P, but not by P alone. The density of 5-HT_{1A} autoreceptor binding in the DRN was decreased by E, P, and E + P treatments. The Kd values obtained from saturation studies were the same for each treatment group. Both the basal and the R-(+)-8-OH-DPAT stimulated [^{35}S]GTP-γ-S binding were decreased during hormone replacement whereas the coupling efficiency between the receptor and G proteins was maintained. EE treatment reduced the level of $G_{\alpha i3}$, but not $G_{\alpha i1}$, $G_{\alpha o}$, and $G_{\alpha z}$ in the DRN. The SERT study demonstrated that the density of SERT binding in the raphe was not changed by one month of E, P, or E + P treatments. However, thirty months of EE treatment increased the SERT protein expression as determined by Western blot analyses. At selective serotonergic target sites in the hypothalamus,

$[^3H]$citalopram binding sites were increased by E, P, and E + P. Finally, E and E + P treatment increased $[^3H]$serotonin uptake in another serotonergic terminal field, the basal ganglia. The Km values for serotonin uptake via SERT were not different among treatments and brain regions. In conclusion, these observations indicated that ovarian hormones may increase serotonin neurotransmission, in part, by downregulating 5-HT_{1A} autoreceptors, 5-HT_{1A} postsynaptic receptors, and the inhibitory G protein utilized for intracellular signal transduction. SERT binding sites and protein expression in the raphe did not reflect SERT mRNA expression during hormone replacement. In addition, SERT binding sites and activity were up-regulated in selective serotonergic terminals, indicating a region specific regulation of SERT trafficking and function. The steroid induced increase in SERT binding in the hypothalamus supports the notion that ovarian hormones increase serotonin neurotransmission and that synaptic serotonin prevents SERT degradation.

CHAPTER 1 Introduction

Since the discovery of serotonin in the brain in 1953, a wealth of information has been generated on the fundamentals of this important neurotransmitter and on the therapeutic actions of serotonergic compounds in treating depression, anxiety, and other mental disorders. This work investigated the role of ovarian steroids in regulating two of the critical components of the serotonin neural system, 5-HT_{1A} receptors and serotonin reuptake transporters (SERT). In the introduction, I will first, give a brief overview of the serotonin hypothesis regarding the etiology of depression. Then, I will review the evidence for the sex difference in serotonin neurotransmission and the importance of the 5-HT_{1A} and SERT. Finally, the hypothesis and aims of this project are stated.

Serotonin and Mental Disorders

Mental illnesses affect an estimated 40 million adults and up to 9 million children and adolescents in this country (Regier et al. 1993). In any given year, 9.5 percent of the population, or about 18.8 million American adults, suffer from a depressive illness. While several theories on the etiology of psychiatric disorders are being developed, evidence is mounting in support of the serotonin hypothesis. Serotonin dysfunction has been implicated in depression, anxiety disorder, aggression, schizophrenia, anorexia, drug abuse, obsessive compulsive disorders, Parkinson's disease, and Alzheimer's disease (Jacobs, Azmitia, 1992). A common symptom among these psychopathological conditions is the depressed mood in the patient. Unbalanced brain chemistry contributes to the onset of affective disorder and evidence for a role of serotonin dysfunction in depression is most convincing.

Depressed patients frequently report increased pain sensitivity, disturbed sleep, changes in appetite, and loss of sexual interest, along with characteristic mood changes. All these symptoms correlate very well with the physiological functions of serotonin. Indeed, low levels of cerebral spinal fluid serotonin and 5-hydroxyindole acetic acid (5-HIAA), the major metabolite of serotonin, in depressed patients have been reported (Mann et al. 1992; Cleare, 1997). In addition, reduced serotonin release and uptake capacity is associated with depression (Rausch et al. 1982; Faludi et al. 1988; Mann et al. 1992; Halbreich, Tworek, 1993; Cleare, 1997). Furthermore, the endocrine marker for central serotonergic tone as indicated by prolactin response to a serotonin releaser, such as fenfluramine, is blunted in depressed patients (Price et al. 1991). Currently available medications for depression, including the selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine and citalopram, the tricyclics, such as imipramine, and the monoamine oxidase (MAO) inhibitors all modify serotonin neural functions. These observations strongly support the importance of serotonin in mood disorders.

Gender Differences

While both genders are burdened by the tremendous personal and financial toll resulting from mental illnesses, the lifetime prevalence of depression in women is twice as high as that in men (Blehar, Oren, 1997; Pearlstein, 1995). Anxiety disorders are nine times higher and eating disorder fifteen times higher in women than in men (Belhar, 1995; Mackenzie, 1986). It is well documented that the anatomy and the circuitry of the brain are sexually dimorphic (Cooke et al. 1998), implying a genetic basis for the different brain functions between sexes. However, the gender differences in mental illnesses are usually not manifested until puberty, suggesting a role of sex hormones. Hormonal changes accompanying female reproductive functions are

thought to cause the sex difference in the psychopathology of depression. For example, depression is associated with the late luteal phase of the menstrual cycle, the perimenopausal stage, and the postpartum period, all of which are physiological states with marked decline of blood estradiol (E) and progesterone (P) (Pearlstein, 1995; Gitlin, Pasnau, 1989; Halbreich et al. 1992).

Limited clinical data support that E has generally favorable effects on mood and cognition, which may be mediated by serotonin. E replacement therapy alleviates depression or anxiety in women (Oppenheim, 1983; Gregoire et al. 1996; Schmidt et al. 2000), and it improves cognitive function (Sherwin, 2000). Additionally, it has been suggested that antidepressants such as SSRIs may be more efficacious in postmenopausal women on hormone replacement therapy than in hormone-naïve patients (Schneider et al. 1997) although this report was limited by a number of factors and no large prospective controlled-studies addressing this issue have been reported. However, the serotonergic agonist, m-CPP, in a challenge test is more potent in the presence of E (Halbreich et al. 1995). In addition, the serotonin releaser, fenfluramine, can cause a greater increase in prolactin secretion in postmenopausal women on E replacement therapy than control subjects (van Amelsvoort et al. 2001). These data indicate that ovarian hormones produced in the periphery may regulate central serotonin function.

Ovarian Hormones and Serotonin

The majority of early studies in rodents examined the serotonin content in brain tissues. Female rats, overall, have higher levels of serotonin in the central nervous system (CNS) than males (Carlsson, Carlsson, 1988; Renner et al. 1985) and several studies reported that ovarian hormones increase the level of serotonin and 5-HIAA in the brain although these findings are confounded with differences in the areas

examined, experimental designs, and interpretation strategies (Bethea et al. 2002a). When serotonin metabolism is blocked by MAO inhibitors such as pargyline, to determine serotonin synthesis rate, a greater accumulation of serotonin occurred with P treatment in E-primed ovariectomized (OVX) rats compared to controls (Walker, Wilson, 1983). Furthermore, P administration partially restored hypothalamic serotonin levels in rats treated with the synthesis inhibitor, para-chlorophenylalanine (pCPA) (Walker, Wilson, 1983).

Recent studies employed microdialysis to measure brain serotonin at discrete locations in freely moving animals. In ewes, E treatment increases serotonin levels in medialbasal hypothalamus (Fabre-Nys et al. 1994). However in rats, ovarian hormones decrease serotonin release in the ventromedial nucleus of the hypothalamus (Farmer et al. 1994; Gundlah et al. 1998). Thus, a species difference in the serotonin response to ovarian hormones is indicated.

In monkeys, the ratio of serotonin and 5-HIAA in the cerebral spinal fluid was increased by the combination of E and P treatment (Schutzer et al. 1997). Furthermore, serum prolactin was stimulated by E + P in the monkey as well (Williams et al. 1985; Sprangers et al. 1990; Bethea et al. 1995). This action of steroids can be blocked by RU486, a specific inhibitor for progesterone receptor, and augmented by fluoxetine, a serotonin uptake inhibitor (Pecins-Thompson, Bethea, 1997). In addition, clinical data accumulated so far supports the positive correlation between central serotonin tone and plasma E and P levels (Bethea et al. 2002a). Together, these observations support the notion that E and P augment serotonin neurotransmission in primates.

Serotonin Neural System

The majority of the brain serotonin is made in neurons located in ten raphe nuclei in the midbrain (Jacobs, Azmitia, 1992). These raphe nuclei contain serotonin

cell groups conventionally designated B1-B9 (Dahlstrom, Fuxe, 1964). The largest raphe nucleus is the dorsal raphe nucleus (DRN) which consists of B6 and B7 serotonin cell groups, followed in size by the median raphe nucleus (MRN), which is composed of B5 and B8 serotonin cell clusters.

Serotonin cells in the raphe nuclei form a descending projection to the structures in the brain stem and the spinal cord as well as ascending projections to the forebrain (Tork, 1990). Serotonin neurons in the DRN send out the fine type-D fibers marked with fusiform butons and the projections from the MRN are composed of thick type-M fibers with the characteristic large butons (Kosofsky, Molliver, 1987; Mulligan, Tork, 1988). These two different types of serotonin fibers enter the medial forebrain bundle together and then, branch out to provide serotonergic innervation to the diencephalon, basal ganglia, limbic system, and cortex. Both D- and M-fiber systems are extensively represented in most areas of the neocortex. However, some of the other brain regions studied thus far have shown a predominance of one system or the other. For example, the striatum and frontal cortex are innervated primarily by D fibers, whereas the hippocampal formation and septum receive input mainly through the M system (Molliver, 1987; Tork, 1990). Other serotonergic projections in the brain include those to the cerebellum and other areas of the midbrain and local connections between the raphe nuclei as well.

The vast distribution of the serotonergic innervation governs a wide array of serotonin functions. Serotonin modifies the stimulatory or inhibitory effect of other neurotransmitters such as glutamate or gamma-amino butyric acid (GABA) (Jacobs, Azmitia, 1992). Thus, brain serotonin has been linked with nearly every physiological and behavioral function regulated by the CNS. Cognition, vigilance state, affect, nociception, food intake, biological rhythm, hormone secretion, and sexual behavior are just a few (Jacobs, Azmitia, 1992). Serotonin also plays a role in synapse formation of

the neuron and brain development (Huang et al. 1997; Whitaker-Azmitia, Azmitia, 1994).

Successful serotonin neurotransmission is accomplished by the coordination of multiple components of the serotonin neural system. For example, the rate of serotonin synthesis is determined by the availability of the serotonin precursor and the activity of tryptophan hydroxylase (TPH). Serotonin storage and release is dependent on cell activity and auto feedback regulation by presynaptic 5-HT_{1A} autoreceptors (see below). SERT proteins on the serotonin cell membrane surface are responsible for the clearance of serotonin out of the extracellular space and the mitochondrial enzyme, MAO, metabolizes serotonin. In addition, the mediators for serotonin actions at a target site are serotonin receptors. Serotonin 5-HT_{1A} and SERT are especially important because of their clinical implications. The experiments in this thesis examined these two pivotal control points of serotonin neurotransmission. The functions of 5-HT_{1A} and SERT, including their impact on intracellular activity, are discussed in the following two sections.

Serotonin 5-HT_{1A} Receptors

Serotonin is utilized by phylogenetically ancient life forms with relatively simple nervous systems as well as human beings, the most complex organism evidenced by evolution. Accordingly, protein receptors for serotonin have been adapting and taking new forms throughout evolution. Therefore, the number of cloned human serotonin receptors has reached fourteen. These serotonin receptors are hitherto grouped into seven families according to their operational (pharmacology), structural (gene and protein sequence), and transductional (intracellular signaling) properties with the guidelines according to the International Union of Pharmacology Receptor Nomenclature Committee (Hoyer et al. 1994).

Overall, variety is the common theme among serotonin receptor systems. Besides the fourteen serotonin receptors (5-$HT_{1A, B, D, E, F}$, 5-$HT_{2A, B, C}$, 5-HT_3, 5-HT_4, 5-$HT_{5A, B}$, 5-HT_6, and 5-HT_7), the existence in the brain of unclassified novel serotonin binding sites, the occurrence of multiple isoforms due to gene splicing (5-$HT_{3, 4, 7}$) or mRNA editing (5-HT_{2C}), and recently reported natural polymorphic variants for many serotonin receptor subtypes all contribute to the biological variation within the serotonin system (Barnes, Sharp, 1999). However, all these individual players function in a well-organized manner as indicated by the highly distinct pattern of distribution in the CNS of each serotonin receptor subtype. All known serotonin receptors can be found post-synaptically while a few (5-$HT_{1A, B}$) are found pre-synaptically on serotonin cells. Some others (5-$HT_{1B, D}$, 5-$HT_{2A, C}$, 5-HT_3, and 5-HT_4) are also located on the nerve terminals of non-serotonin neurons as heteroceptors to regulate neurotransmitter release. All serotonin receptors known to us belong to the G protein coupled receptor superfamily with the exception that 5-HT_3 is a ligand-activated cation channel. The coordinated recruitment of various serotonin receptors accomplishes the physiological functions of serotonin in the brain. Dysfunction of serotonin receptors, therefore, contributes to various mental disorders.

Serotonin 5-HT_{1A} receptors belong to the 5-HT_1 family of serotonin receptors along with 5-HT_{1B}, $_{1D}$, $_{1E}$, and $_{1F}$ receptors (Barnes, Sharp, 1999). In general, members of the 5-HT_1 receptor family have higher affinity for the endogenous substrate, serotonin, than members of any other serotonin receptor families. Consequently, when a cell co-expresses multiple serotonin receptor subtypes, 5-HT_1 receptors probably mediate the sensitive response to a low level of transmitter exposure while other serotonin receptors may come into play later on when synaptic serotonin reaches a sufficient concentration. A selective agonist for 5-HT_{1A} receptors is 8-OH-DPAT and a selective antagonist is Way-100635. 5-HT_{1A}, $_{1B}$ and $_{1D}$ also have high affinity for the

synthetic compound, 5-CT, and β_2 adrenoceptor ligands such as pindolol, but 5-HT_{1E} and $_{1F}$ do not.

All five 5-HT_1 receptor subtypes have seven transmembrane domains characteristic of G protein coupled receptors (Barnes, Sharp, 1999). The sequence homology between 5-HT_{1A} and other members of this family is approximately 40%, similar to the homology among 5-HT_{1A} receptors and members of other serotonin receptor family. However, 5-HT_{1B}, $_{1D}$, $_{1E}$, and $_{1F}$ have 77% homology with each other within the transmembrane regions. Although other second messenger systems, such as intracellular Ca^{2+} and phospholipase C, etc., have been linked with 5-HT_1 receptors in transfected cell systems, the endogenous signaling system coupled to 5-HT_1 receptors is Gi/o-adenylate cyclase-K^+ channels.

Specifically, in the case of 5-HT_{1A} receptors, the second and third intracellular loops of the receptor make contact with G proteins (Varrault et al. 1994; Ou et al. 2001). Ligand binding induces conformational changes in 5-HT_{1A} receptors, which subsequently activate G proteins. The alpha subunit of the G-proteins coupled with 5-HT_{1A} receptors, such as $G_{\alpha i3}$, $G_{\alpha i2}$, or $G_{\alpha i1}$, inhibit the activity of adenylate cyclase, a family of proteins that generates an important intracellular signaling molecule, cyclic adenosine monophosphate (cAMP) (Raymond et al. 1999). In addition, the activation of G_α releases $\beta\gamma$ subunits of the G protein complex, which interact with G protein-gated inwardly rectifying potassium channels and open the channel (Andrade, Nicoll, 1987; Zgombick et al. 1989; Doupnik et al. 1996). The combination of decreased levels of cytosolic cAMP and opening of K^+ channels decreases cell activity and cell firing (Raymond et al. 1999). Currently available antidepressant medications, such as SSRIs, must be taken regularly for 3 to 4 weeks (in some cases, as many as 8 weeks) before the full therapeutic effect occurs. This delay is mainly due to the activation of 5-HT_{1A} autoreceptors on serotonin cells by elevated extracellular serotonin during initial

drug treatment (Blier et al. 1998; Briner, Dodel, 1999; Hjorth, Auerbach, 1996; Hjorth et al. 2000). The activation of this auto-inhibitory receptor prevents further serotonin release. SSRIs manifest their efficacy when 5-HT_{1A} is desensitized (Hjorth, Auerbach, 1996).

The intracellular signals or physiological responses, such as the inhibition of cytosolic cAMP levels produced by 5-HT_{1A} activation progressively diminishes during continuous stimulation of the receptor. This desensitization process of 5-HT_{1A} receptors is mediated by multiple mechanisms including phosphorylation of 5-HT_{1A} receptors by protein kinases C (PKC), A and G protein related kinases (Raymond, 1991; Liu, Albert, 1991; Raymond, Olsen, 1994; Nebigil et al. 1995). Recently, the 5-HT_{1A} antagonist, pindolol, has been supplemented by an SSRI regimen with success in expediting the onset of the antidepressant action (Artigas et al. 1996; Sacristan et al. 2000).

5-HT_{1A} receptors are found pre-synaptically on serotonin cell bodies and dendrites (Kia et al. 1996; Azmitia et al. 1996a). Serotonin target cells in frontal cortex express post-synaptic 5-HT_{1A} receptors, with the densest population found in the hippocampus and septum (Barnes, Sharp, 1999). 5-HT_{1A} agonists inhibit serotonin release but increase acetylcholine and norepinephrine release. They also induce hyperphagia, hypothermia, and the elevation of plasma ACTH, coritcosteroids, and growth hormone (Barnes, Sharp, 1999). Both pre- and post-synaptic 5-HT_{1A} receptors have been implicated in affective disorders (Stockmeier, 1997; Stockmeier et al. 1997; Luscombe et al. 1993). 5-HT_{1B} and $_{1D}$ are found pre-synaptically and post-synaptically in the basal ganglia, substantia nigra, and to a lesser degree in the hippocampus and raphe. These two receptors inhibit synaptic terminal release of serotonin, dopamine, acetylcholine, glutamate, and GABA. 5-$HT_{1B, D}$ agonists may stimulate locomoter activity and hypophagia (Barnes, Sharp, 1999). 5-$HT_{1E, F}$ receptors are found in the

entorhinal cortex and basal ganglia. Little is known about the physiological functions of these two subtypes.

Serotonin Reuptake Transporter

The primary means for serotonin cells to terminate synaptic serotonin transmission is via SERT. SERT is predominantly located pre-synaptically in proximity of serotonin nerve terminals (Lesch et al. 1993). Translocation of serotonin from the extracellular space back to the inside of serotonin cells is the major task of SERT (Blakely et al. 1994). SERT proteins are also expressed on serotonin cell bodies and dendrites for uptake of serotonin released somato-dendritically (Lesch et al. 1993; Blakely et al. 1994). SSRIs bind with SERT protein, block the transport of serotonin through SERT and thereby, prolong the actions of serotonin at pre- and postsynaptic receptor sites. Therefore, SSRIs are effective antidepressants and anxiolytics.

SERT belongs to the Na^+/Cl^--dependent cotransporter family (Blakely et al. 1994). The influx of extracelluar Na^+ ion into the cell down its concentration gradient generates the energy for inward transfer of serotonin. In addition to Na^+ ions, extracelluar Cl^- ions are also necessary for serotonin transport. Na^+, Cl^-, and protonated serotonin bind with SERT and form a quaternary complex, which then undergoes a conformational change to release serotonin molecules and ions into the cytoplasm. Subsequently, intracellular K^+ facilitates the reorientation of the vacant serotonin binding sites on the carrier for another transport cycle.

The human SERT protein has six phosphorylation sites that are acted upon by PKC and PKA (Ramamoorthy et al. 1998; Blakely et al. 1998). The phosphorylation of SERT promotes transporter internalization and decreases its membrane expression and serotonin uptake (Ramamoorthy, Blakely, 1999). The regulation of SERT through

this mechanism is usage dependent. That is when serotonin molecules pass through the portal of SERT proteins, the phosphorylation and internalization of SERT is inhibited whereas when the passage of serotonin through SERT is blocked by SSRIs and other compounds such as cocaine and methamphetamine, the phosphorylation and internalization of the transporter accelerates (Ramamoorthy, Blakely, 1999). Thus, there can be multiple points to regulate SERT activity by ovarian steroids, such as at the level of gene expression, translation, protein phosphorylation, trafficking, and metabolism.

Non-human Primate Model

Non-human primates offer a unique model for investigating the regulation of the serotonin system by ovarian hormones. They have a menstrual cycle identical to that of humans. Their CNS is highly developed and comparable to that of humans and they manifest complex social interactions. The Bethea laboratory has documented estrogen receptor β and progestin receptor expression in serotonin neurons in monkeys, unequivocally establishing that serotonin neurons are target cells for ovarian steroids (Gundlah et al. 2000; Bethea, 1993). Estrogen receptors β and progestin receptors are transcription factors that, upon activation by their cognate hormones, regulate the expression of target genes. E, via estrogen receptor β, induces progestin receptor expression in serotonin cells in monkeys (Bethea, 1994).

The expression pattern of steroid receptors in serotonin neurons of monkeys is different than that of other species studied so far. In rats, estrogen receptor β, but not progestin receptor, is found in the serotonin neurons (Shughrue et al. 1997; Alves et al. 1998). In fact, progestin receptor is expressed in interneurons adjacent to serotonin neurons in rats. In mice, estrogen receptor β, another estrogen receptor, α, and progestin receptor are all located within serotonin neurons (Mitra et al. 2001; Alves et

al. 2000). On the contrary, estrogen receptor α is not expressed in monkey serotonin cells. In conclusion, different combinations of steroid receptors within the serotonin neurons in different species indicate a species difference in the actions of ovarian hormones in the serotonin system and non-human primates provide a unique model for studying the interaction of E, P, and serotonin.

Statement of Purpose

It was previously reported that ovarian hormone replacement in OVX macaques regulates gene expression in the serotonin neurons. For example, E with or without P increased the gene and protein expression of TPH, the rate-limiting enzyme for serotonin synthesis (Pecins-Thompson et al. 1996; Bethea et al. 2000), but decreased the expression of 5-HT_{1A} autoreceptor (Pecins-Thompson, Bethea, 1999) and SERT mRNA (Pecins-Thompson et al. 1998) in the DRN of macaques. The overall hypothesis of this project is that the down-regulation of 5-HT_{1A} and SERT gene expression is manifested at the functional protein level during hormone replacement. E and P thereby may stimulate serotonin neurotransmission via the disinhibition of serotonin neurons by downregulating 5-HT_{1A} autoreceptors and via prolonging actions of synaptic serotonin by decreasing reuptake.

Both 5-HT_{1A} and SERT are critical for serotonin neurotransmission and psychopathology of depression. SERT is the site of action of widely prescribed SSRI antidepressants and the combination of 5-HT_{1A} antagonists with SSRIs outperforms SSRIs alone in treating depression symptoms (Artigas et al. 1996; Sacristan et al. 2000). This study is to determine the ovarian steroid regulation of 5-HT_{1A} (Chapter 3, Aim 1 and 2, see below) and SERT (Chapter 4, Aim 3 and 4) at the functional protein level.

Specifically, **Aim 1** will use receptor autoradiography to measure and compare pre- and postsynaptic 5-HT_{1A} binding sites in OVX macaques from control, E treated, P treated, and E + P treated groups. **Aim 2** will subsequently determine and compare the coupling of 5-HT_{1A} receptors with G proteins in animals from different treatment groups by measuring 5-HT1A receptor activated [^{35}S]GTP-γ-S binding. **Aim 3** will use receptor autoradiography to determine SERT density in different treatment groups by measuring the binding density of an SSRI, [^{3}H]citalopram at various brain areas. **Aim 4** will measure the uptake of serotonin in synaptosomal preparations from SERT concentrated brain regions to determine whether female hormones regulate the function of SERT.

The results of this study will further our knowledge on how female hormones affect serotonin uptake through the SERT and how the negative feedback on serotonin cell activity through 5-HT_{1A} autoreceptors are regulated by ovarian steroids. This information should be useful to understand the roles of ovarian steroids in serotonin neural functions and to benefit the search for effective treatment regimens for mental disorders such as depression.

CHAPTER 2 General Methods

Reagents

[^{3}H]8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, 124.9-135 Ci/mmol), [35S]Guanosine 5'-(γ-thio)triphosphate (GTP-γ-S, 1250 Ci/mmol), [^{3}H]Citalopram (82 Ci/mmol) and [^{3}H]serotonin (25.5 Ci/mmol) were obtained from Perkin Elmer Life Sciences (Boston, MA). Mouse monoclonal anti-human SERT antibody (ST51-2) was from Mab Technologies, Inc. (Stone Mountain, GA). Rabbit anti-$G_{\alpha i3}$ poylclonal antibody was from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-$G_{\alpha i1}$, -$G_{\alpha o}$, and -$G_{\alpha z}$, polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse and goat anti-rabbit antibodies conjugated to horseradish peroxidase (HRP) were purchased from ICN Biomedicals, Inc. (Aurora, Ohio) and Chemicon International (Temecula, CA), respectively. Unlabeled citalopram was a generous gift of Lundbeck (Copenhagen, Denmark). All other reagents, unless otherwise stated, were from Sigma-Aldrich (St. Louis, MO).

Animals

This study was approved by the Institutional Animal Care and Use Committees of Oregon Regional Primate Research Center (ORPRC) and the Bowman Gray School of Medicine. Animals were euthanized according to procedures recommended by the Panel on Euthanasia of the American Veterinary Association.

Twenty adult female rhesus monkeys *(Macaca mulatta)* were OVX and twelve out of the twenty were also hysterectomized in other research programs according to standard veterinary procedures by the surgical personnel of ORPRC. Approximately 2-6 months after finishing other protocols, the animals were assigned to this study. The rhesus macaques were born and reared in Oregon and were in good health. The

animals were all young adults from 7 to 12 years old and weighed between 4.5 and 7.5 kg.

The OVX rhesus macaques received either an empty Silastic capsule for 28 days (OVX controls), an E-filled capsule for 28 days (E treated), a P-filled capsule for 28 days (P treated) or an E capsule for 14 days supplemented with a P capsule for an additional 14 days (E + P treated). Each treatment group contained five animals.

Treatment with E and P as described above has been shown to cause uterine endometrium differentiation in a manner similar to the normal 28-day menstrual cycle (Brenner, Maslar, 1988). The E regimen has also been shown to induce nuclear progestin receptor expression in numerous target organs including the brain (Bethea et al. 1992; Bethea, 1994) and the addition of P to the E regimen stimulates prolactin secretion (Williams et al. 1985; Sprangers et al. 1990; Bethea et al. 1992).

Cynomolgus macaques (*Macaca fasicularis*) were imported directly from Indonesia (Institut Pertanian Bogor, Bogor, Indonesia) to the Department of Comparative Medicine, Wake Forest University, Winston-Salem, NC. For a total of 34 months, all animals were fed a moderately athrogenic diet (40% of calories were from fat and 0.28 mg cholesterol/kcal) (Adams et al. 1990). Monkeys lived in social groups consisting of four to six animals. They were ovariectomized and consumed the atherogenic diet for a 4-month pre-experimental period. Monkeys were then assigned to various hormonal treatment groups according to a stratified randomization scheme. For this study, the midbrain was obtained from three OVX control animals and three OVX animals treated with conjugated equine estrogen (EE, Premarin, Wyeth-Ayerst, Princeton, NJ) in the diet for 30 months (Adams et al. 1997). The dosage of EE was adjusted for body size and metabolic rate to approximate a serum concentration of EE in the treated monkeys to that of women taking the same compound (Clarkson et al. 1990).

Rhesus and cynomolgus macaques are two closely related species. They have similar menstrual cycles and are cross-fertile. Immunocytochemistry studies have reported that nuclear receptors for ovarian steroid are present in serotonin neurons of both species (Bethea, 1993).

Surgery and Steroid Treatments of Rhesus Macaques

Silastic capsules were placed in the periscapular area under ketamine anesthesia (ketamine HCl, 10 mg/kg, I.M.; Fort Dodge Laboratories, Fort Dodge, IA). The E treated monkeys were implanted (S.C.) with one 4.5 cm E-filled Silastic capsule (inner diameter, 3.35 mm, outer diameter, 4.65 mm; Dow Corning, Midland, MI). The capsule was filled with crystalline estradiol [1,3,5(10)-estratrien-3,17-β-diol, Steraloids, Wilton, NH). The P treated monkeys were implanted with one 6.0 cm Silastic capsule containing crystalline progesterone (4-pregnen-3,20 dione, Steraloids). The E + P treated group received a 4.5 cm E-filled capsule, and 14 days later, received one 6.0 cm P-filled capsule for an additional 14 days. OVX monkeys implanted with empty 4.5 cm Silastic capsules were used as the control group.

Tissue Harvest and Cryosection for Autoradiography

The rhesus monkeys were euthanized at the end of the treatment period according to the procedures recommended by the Panel on Euthanasia of the American Veterinary Association. Each animal was sedated with ketamine (10 mg/kg I.M.), given an overdose of pentobarbitol (25 mg/kg, I.V.), and exsanguinated by severance of the descending aorta. The left ventricle of the heart was cannulated and the head of each monkey was perfused with 1 L of 0.25 M sucrose in 0.05 M Tris buffer, pH 7.4, containing 5000 unit/L heparin and 2 L of 0.5 M sucrose in 0.05 M Tris buffer, pH 7.4. The brain was removed and dissected. Hypothalamic and midbrain pontine

blocks were frozen in isopentane cooled to –55°C and stored at -80°C until sectioning that occurred within 2 months of storage. Coronal sections (10 µm) were cut on a cryostat at –20 to –22°C, thaw-mounted on Superfrost Plus Slides (Fisher Scientific, Santa Clara, CA), dehydrated under vacuum at 4°C for 2 hr and then frozen at –80°C until processing for binding studies. Every seventeenth section at an interval of 170 µm was stained with thionin for morphological references and anatomical orientation (Paxinos et al. 2000).

Densitometry Analysis of Autoradiograms

Densitometry of the autoradiograms generated from binding studies was performed using NIH image on a Macintosh computer. Two measurements were generated from the autoradiograms, which required the operator to circle the anatomical area of interest. The first measurement was the average gray scale optical density, obtained by subtracting the background optical density value from the total optical density value of the region of interest. This indicates the relative intensity of the signal. The second measurement yielded the average positive pixel area, obtained by setting a threshold for positive signals above the background level (the same setting was used for all animals). The positive pixels indicate the area covered by the signal. Thus, a decrease in this parameter may represent a decrease in the number of cells above the threshold for detection. The optical density and positive pixel area reflect different aspects of protein binding signals although they should, in general, change in the same direction for a defined area.

Tissue Preparation for Western Blot Analysis

Dorsal raphe tissue extracts were obtained from six OVX adult female cynomolgus macaques for Western blot analyses of G protein subunits. At the end of

the treatment protocol described above, the monkeys were anaesthetized deeply with pentobarbital (30 mg/kg, I.V.); the cranium was retracted and the brain was removed for dissection. The individual brain blocks, including a mid- and hindbrain section, were sealed in plastic bags, immersed in liquid nitrogen, and then stored at –80°C until microdissection of the midbrain. Detailed tissue preparation for Western analysis has been published (Bethea et al. 2000).

Western Blot Analysis

Monkey midbrain pontine blocks containing the DRN were microdisected and hand homogenized in 50 mM Tris and 20 mM β-mercaptoethanol, pH 7.5 (Bethea et al. 2000). The homogenates were subjected to centrifugation at 12,000 X g at 4°C for 10 minutes. Pellets containing membrane bound proteins were obtained and resuspended in Tris (10 mM) and EDTA (1 mM), pH 7.2, containing leupeptin (1 μg/ml), Trypsin inhibitor (1 mg/ml), O-phenanthroline (1 mM), iodoacetamide (1 mM), PMSF (250 mM), and pepstatin A (1 μM) and further homogenized with a hand-held pestle mortar (Fisher Scientific). The concentrations of the total protein in the homogenates were determined with the Bio-Rad (Hercules, CA) protein determination reagent according to Bradford (Bradford, 1976). Samples containing 50 μg of total protein from each animal were dissolved with 10% SDS containing 4% β-mercaptoethanol at 80°C for 15 min and heated at 90°C for 10 min before loading onto a vertical mini gel system.

Hormone Assays

Concentrations of serum E, P, and prolactin of the rhesus macaques were measured in samples obtained at necropsy. Radioimmunoassays were performed in the Endocrine Service Core at ORPRC as described previously (Resko et al. 1974;

Resko et al. 1975; Bethea, Papkoff, 1986). Concentrations of serum E in cynomolgus macaques were measured at Wake Forest University (Bethea et al. 2000).

Statistics

For autoradiography, measurements from four to six sections (300 μm apart) of each area were examined to yield an average for each animal. The optical density and positive pixel area of binding signals on the autoradiograms were within the linear range of standards. There were five animals in each treatment. Average values from each treatment group were compared by unpaired one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc pairwise comparisons. For Western blot analyses, the density of signal bands from OVX and EE treated groups were compared by two-tailed Student's t tests. Steroid hormone concentrations were compared with one-way ANOVA and Student-Newman-Keuls post hoc pairwise comparisons. Saturation and competition curves from binding and uptake assays were analyzed with a nonlinear regression curve-fitting program. The maximal transport (Vmax) and Michaelis-Menton constant (Km) of serotonin uptake among treatments and brain regions were compared with two-way ANOVA. When a difference among treatment groups was indicated by the two-way ANOVA, then one-way ANOVA and post hoc pairwise comparisons were performed to further determine the differences between treatments at each brain area. All analyses and statistical comparisons between treatments were conducted with Prism 3.0 (San Diego, CA). A confidence level of $p < 0.05$ was considered significant.

CHAPTER 3 Ovarian Steroid Regulation of 5-HT_{1A} Receptor Binding and G protein Activation in Female Monkeys

ABSTRACT

Serotonin 5-HT_{1A} receptors play an important role in serotonin neurotransmission and mental health. We previously demonstrated that estradiol (E) and progesterone (P) decrease 5-HT_{1A} autoreceptor mRNA levels in macaques. In this study, we questioned whether E and P regulate 5-HT_{1A} binding and function and G_{α} subunit protein expression. Quantitative autoradiography for 5-HT_{1A} receptors and G proteins using [^{3}H]8-OH-DPAT and [^{35}S]GTP-γ-S, respectively, was performed on brain sections of rhesus macaques from four treatment groups: ovariectomized controls (OVX), E (28 d), P (28 d), and E (28 d) plus P (the last 14 d) treated. Western blot analysis for G_{α} subunits was performed on raphe extracts from cynomolgus macaques that were OVX or OVX treated with equine estrogens (EE, 30 months). In the hypothalamus, E or E + P but not P alone decreased postsynaptic 5-HT_{1A} binding sites. In the dorsal raphe nucleus (DRN), E, P, and E + P treatments decreased 5-HT_{1A} autoreceptor binding. The Kd values for 8-OH-DPAT were the same for each treatment group. Both the basal and the R-(+)-8-OH-DPAT stimulated [^{35}S]GTP-γ-S binding were decreased during hormone replacement whereas the coupling efficiency between the receptor and G proteins was maintained. Finally, EE treatment reduced the level of $G_{\alpha i3}$, but not $G_{\alpha i1}$, $G_{\alpha o}$, and $G_{\alpha z}$ in the DRN. In conclusion, these observations suggest that ovarian hormones may increase serotonin neurotransmission, in part, by decreasing 5-HT_{1A} autoreceptors, 5-HT_{1A} postsynaptic receptors, and the inhibitory G proteins for intracellular signal transduction.

A body of evidence suggests that actions of the ovarian hormone, estradiol (E), on mood and cognitive function may be mediated by the serotonin neural system (McEwen, 1999; Mann, 1999). We have found that E, with or without progesterone (P), acts on gene expression in serotonin neurons in a manner that could increase serotonin neurotransmission. That is, E ± P treatment of ovariectomized (OVX) macaques increased tryptophan hydroxylase gene and protein expression (Pecins-Thompson et al. 1996; Bethea et al. 2000), but decreased the expression of serotonin reuptake transporter (Pecins-Thompson et al. 1998) and 5-HT_{1A} autoreceptor mRNAs (Pecins-Thompson, Bethea, 1999) in the dorsal raphe nucleus (DRN) of macaques. It is necessary, however, to know whether these documented changes in gene expression have functional consequences in the serotonin neural system.

Serotonin 5-HT_{1A} receptors, either pre- or postsynaptically, play a pivotal role in serotonin neurotransmission (Raymond et al. 1999). The presynaptic autoreceptor, on the soma and dendrites of serotonin neurons, binds serotonin in the extracellular space and decreases neuronal firing and serotonin release (Sprouse, Aghajanian, 1987; Blier, de Montigny, 1987; Azmitia et al. 1996a). This ultra-short loop feedback mechanism is thought to cause a delay in the onset of efficacy of antidepressant drugs (Hjorth, Auerbach, 1996; Hjorth et al. 2000). In depressed patients, 5- HT_{1A} autoreceptor levels are elevated (Stockmeier et al. 1998) and supplementation of antidepressant therapy with 5-HT_{1A} antagonists, such as pindolol, outperforms selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine alone (Artigas et al. 1996; Sacristan et al. 2000).

The reported effects of ovarian hormones on 5-HT_{1A} receptor binding in the rodent are not consistent. Depending on the area examined and the treatment regimen, postsynaptic 5-HT_{1A} receptor binding sites have been reported to increase (Flugge et al. 1999), decrease (Osterlund et al. 2000), or not change (Clarke, Maayani, 1990;

Frankfurt et al. 1994). Studies on ovarian steroid regulation of 5-HT_{1A} autoreceptor binding in rodents are lacking. We previously demonstrated that E ± P treatment of OVX macaques for one month decreased 5-HT_{1A} autoreceptor mRNA in the DRN (Pecins-Thompson, Bethea, 1999). However, no change was observed in the postsynaptic 5-HT_{1A} receptor mRNA level in the hypothalamus (Gundlah et al. 1999). The effect of E or P on the binding activity of 5- HT_{1A} receptors in primates is unknown.

The 5-HT_{1A} receptor causes activation of the inhibitory G protein of $G_{i/o/z}$ families (Raymond et al. 1999). The association of guanosine triphosphate (GTP) molecules with α subunits of the G protein complex subsequently triggers cytosolic processes that decrease cAMP levels whereas $\beta\gamma$ subunits cause the opening of G protein-gated inwardly rectifying K^+ channels on cell membranes and thereby inhibit cell-firing (Raymond et al. 1999). E administration decreases the inhibition of 8-OH-DPAT, a potent 5-HT_{1A} agonist, on serotonergic cell firing in the DRN of rats (Lakoski, 1988). Other studies demonstrate that E desensitizes 5-HT_{1A} mediated release of stress hormones such as corticosteroids by decreasing the level of subunits of G proteins (Raap et al. 2000). Clinical data indicate that subunits of G proteins are dysfunctional in psychiatric patients with bipolar disorders, depression, and anxiety (Vawter et al. 2000; Manji, Lenox, 2000). However, it is not known whether E and P affect 5-HT_{1A} function and/or coupling to G proteins in primates. Therefore, we questioned whether E, P, or E + P would alter (1) post- and presynaptic 5- HT_{1A} receptor binding, (2) basal and 8-OH-DPAT stimulated [^{35}S]GTP-γ-S binding, and (3) G_α protein expression.

In this study, quantitative autoradiography was used to measure 5-HT_{1A} receptor-binding sites in hormone treated macaques and untreated OVX controls. Postsynaptic 5-HT_{1A} binding sites were determined in the hypothalamus and

presynaptic 5-HT_{1A} binding sites were determined in the DRN. In addition, the effect of E and P on 5-HT_{1A} autoreceptor function was examined by determining the level of [^{35}S]GTP-γ-S binding with or without stimulation by R-(+)-8-OH-DPAT. Lastly, the generous donation of fresh-frozen midbrains from cynomolgus macaques maintained on long-term hormone replacement therapy enabled the examination of G protein expression.

SPECIFIC MEHTODS

[^{3}H]-8-OH-DPAT Binding

5-HT_{1A} binding experiments were performed according to Verge et al (Verge et al. 1986). Briefly, sections were brought to room temperature in a desiccator and preincubated in the preincubation buffer (170 mM Tris and 4 mM $CaCl_2$, pH 7.6) at 22°C for 30 min. Then, sections were incubated with 2 nM [^{3}H]-8-OH-DPAT in assay buffer (the preincubation buffer supplemented with 0.01% L–ascorbic acid, 10 μM pargyline, and 10 μM fluoxetine) for 1 hr followed by two rinses in the preincubation buffer for 4 min each at 4°C. Slides were then dipped in distilled water at 4°C for 3 sec and dried rapidly with cold air. Non-specific binding was assessed on adjacent sections with the addition of 2 μM serotonin. Saturation studies were performed by incubating serial sections with [^{3}H]-8-OH-DPAT at seven concentrations ranging from 0.1 nM to 8 nM.

Matching sections from OVX controls, E treated, P treated and E + P treated monkeys were processed in the same experiment and exposed to ^{3}H-sensitive ultra films along with ^{3}H autoradiographic micro-scales (Amersham, Arlington Heights, IL) for 30 days. Films were developed in Kodak developer for 5 min and fixed for 8 min. Autoradiograms were digitized with a SONY CCD video camera. Each film was calibrated with ^{3}H autoradiographic micro-scales.

In addition, twelve concentrations ranging from 0.1 nM to 300 μM of unlabeled serotonin, Way-100635 (5-HT_{1A} antagonist), L-694247 (5-$HT_{1B/D}$ antagonist; Tocris, Ballwin, MO), and ketanserin (5-HT_{2A} antagonist) were used to compete for [^{3}H]-8-OH-DPAT binding on serial sections. In these competition studies, sections were scraped off the slides, after washing, with GF/C Glass Microfiber filters (Whatman, England), equilibrated with 3 ml of BD ScintiVerse (Fisher Chemicals, Fair Lawn, NJ) for 3 hrs, and counted on a Tri-carb 1500 scintillation counter (Packard Instrument Co., Meridan, CT).

G Protein Autoradiography Using [^{35}S]GTP-γ-S

[^{35}S]GTP-γ-S binding experiments were performed according to Dupuis et al (Dupuis et al. 1999) and Sim et al (Sim et al. 1997). Briefly, sections were brought to room temperature in a desiccator and preincubated in the assay buffer (in mM, 50 HEPES, 50 $NaCl_2$, 3 $MgCl_2$, and 0.2 EGTA, pH 7.4) at 25°C for 10 min. Then, sections were incubated with 2 mM GDP in the assay buffer at 25°C for 15 min followed by incubation with 0.04 nM [^{35}S]GTP-γ-S in the assay buffer supplemented with 2 mM GDP, 0.2 mM dithiothreitol (Roche Molecular Biochemicals, Indianapolis, IN), and 10 mU/ml adenosine deaminase at 25°C for 90 min. At the end of the incubation, reagents were washed off the sections by rinsing with 50 mM HEPES, pH 7.4 twice for 2 min each and dipping in distilled water at 4°C. Sections were rapidly dried with cool air. The basal and stimulated binding were defined in the absence or presence of 1 μM of R-(+)-8-OH-DPAT, respectively, during the incubation with [^{35}S]GTP-γ-S. Non-specific binding was defined on adjacent sections by the addition of 10 μM of unlabeled GTP-γ-S. Saturation studies were performed by incubating serial sections with [^{35}S]GTP-γ-S at concentrations ranging from 4 to 400 pM. Also, 0.1 and 1 μM Way-100635 and phentolamine (α adrenergic blocker) were used to

block the R-(+)-8-OH-DPAT stimulated [^{35}S]GTP-γ-S binding. In addition, nine concentrations of R-(+)-8-OH-DPAT up to 3 μM were used to stimulate [^{35}S]GTP-γ-S binding on serial sections to evaluate the potency of this 5-HT$_{1A}$ agonist in the macaque.

Matching sections from OVX control animals, E treated, P treated and E + P treated monkeys were processed in the same experiment and exposed to Kodak Biomax MR films along with ^{14}C autoradiographic micro-scales (Amersham, Arlington Heights, IL) for 3 to 6 days. Films were developed in Kodak developer for 2 min and fixed for 5 min. Autoradiograms were digitized with a SONY CCD video camera. Densitometry was performed using NIH Image software as described above. Each film was calibrated with ^{14}C autoradiographic micro-scales.

Western Analysis for G Protein Subunits

Western blot analyses were performed according to the modified procedures of Mullaney and Miligan (Mullaney, Miligan, 1990) with blotting buffer containing 25 mM of Tris base and 192 mM of glycine. The nitrocellulose membranes (Osmonics, Westborough, MA) were blocked in 5% non-fat dry milk for 45 min before incubating with antibodies against G_α subunits at 4°C overnight. The dilutions for rabbit anti-$G_{\alpha i3}$, $G_{\alpha i1}$, $G_{\alpha o}$, and $G_{\alpha z}$, were 1:1600, 1:150, 1:700, and 1:200, respectively. The following morning, the blots were washed in saline and 0.05% tween-20 (Bio-Rad) and incubated with 1:2000 of goat anti-rabbit antibody conjugated to HRP at room temperature for 2 hr and then developed with Supersignal chemiluminesence kits (Pierce, Rockford, IL) followed by exposing to Kodak X-OMAT AR films. Densitometric analysis of signal bands was performed with NIH Image Gel Plotting software.

RESULTS

Distribution Pattern and Regulation of [^{3}H]8-OH-DPAT Binding in the Monkey Hypothalamus

The anatomical distribution of hypothalamic postsynaptic 5-HT$_{1A}$ receptors labeled with [^{3}H]8-OH-DPAT matched the distribution pattern of 5-HT$_{1A}$ mRNA in the hypothalamus observed previously (Gundlah et al. 1999). A discrete pattern of [^{3}H]8-OH-DPAT binding in the hypothalamus was observed in the vertical limb of diagonal band of Brocca (DB), preoptic area (POA), supraoptic (SON), paraventricular (PVN), periventricular (PEV), ventromedial nucleus (VMN), and the dorsal medial hypothalamus (DMH) (Figure 3-1).

Average optical density values from four to six levels of each hypothalamic nucleus (n = 5/treatment) were compared with ANOVA. 5-HT$_{1A}$ binding sites were significantly decreased by E or E + P, but not by P alone compared to the OVX control animals in the DB, PVN, PEV, VMN, and DMH (Figure 3-2). Positive pixel areas reflected optical density values in each hypothalamic region examined. In the SON and POA, optical density values and pixel areas of [^{3}H]8-OH-DPAT binding were not different among treatment groups.

Distribution Pattern and Regulation of [^{3}H]8-OH-DPAT Binding in the Monkey Raphe

[^{3}H]8-OH-DPAT labeling on monkey midbrain sections matched the distribution pattern of 5-HT$_{1A}$ autoreceptor mRNA (Pecins-Thompson, Bethea, 1999), i.e., robust in the dorsal raphe, moderate in the median raphe, and light in the periaquiductal gray (Figure 3-3). Specific [^{3}H]8-OH-DPAT binding was saturable (Figure 3-4). Kd values (nM ± SEM) for the radioligand on monkey midbrain

sections equaled 3.06 ± 2.26, 1.86 ± 2.25, 3.21 ± 2.88, and 2.66 ± 2.68 in OVX, E, P, and E+P treated groups, respectively (not different, $p > 0.05$). Unlabeled serotonergic compounds blocked the binding of [^{3}H]8-OH-DPAT with the following IC_{50} rankings: Way-100635 < 5-HT < L-694,247 << Ketanserin, agreeing with the literature (Boess, Martin, 1994; Nelson, 1991)

E, P, and E + P treatments all significantly reduced the level of 5-HT_{1A} autoreceptor binding sites in the DRN compared to the OVX controls (Figure 3-5). Average optical density values from five levels of the DRN (n = 5/treatment) were significantly decreased by E, P, and E + P. Pixel areas reflected the optical density values. Compared to the OVX control animals, the positive pixel area representing [^{3}H]8-OH-DPAT binding in the DRN was significantly decreased by all three treatments.

[^{35}S]GTP-γ-S Binding in the DRN

The distribution pattern of [^{35}S]GTP-γ-S binding sites in the DRN matched with that of [^{3}H]8-OH-DPAT binding (Figure 3-6). Average optical density values and pixel area measurements of [^{35}S]GTP-γ-S binding sites on five levels of the DRN are shown in figure 3-7. Compared to OVX control animals, the optical density values representing the basal and stimulated [^{35}S]GTP-γ-S binding were both significantly decreased by E, P, and E+P compared to the control group. The positive pixel areas reflected the optical density values and were significantly decreased in all treatment groups.

The apparent Kd values (nM ± SEM) for the basal [^{35}S]GTP-γ-S binding equaled 0.148 ± 0.022, 0.130 ± 0.019, 0.153 ± 0.024, and 0.106 ± 0.017 whereas the Kd values for the stimulated binding equaled 0.122 + 0.016, 0.115 ± 0.015, 0.119 ± 0.016, and 0.093 ± 0.015 in OVX, E, P, and E + P treated groups, respectively (not

different, $p > 0.05$). These values indicate that the affinity of the radioligand with G proteins on monkey midbrain sections was not affected by any of the treatments.

In addition, adjacent midbrain sections were incubated with increasing concentrations of R-(+)-OH-DPAT to stimulate $[^{35}S]$GTP-γ-S binding. The concentration of R-(+)-OH-DPAT (nM ± SEM) producing half-maximal stimulation equaled 3.09 ± 0.33, 1.96 ± 0.46, 3.51 ± 0.78, and 3.55 ± 1.10 in OVX, E, P, and E + P treated groups, respectively (not different, $p > 0.05$). Thus, the potency of R-(+)-OH-DPAT to stimulate $[^{35}S]$GTP-γ-S binding was the same in each treatment group.

Lastly, the coupling efficiency between $5\text{-}HT_{1A}$ and G proteins was calculated as (Stimulated – Basal) / Basal X 100 % and averaged for each treatment group. In OVX, E, P, and E + P treated groups, the percentage increase from basal to stimulated $[^{35}S]$GTP-γ-S binding as derived from optical density values equaled 54.64 ± 3.42, 62.02 ± 8.09, 57.04 ± 4.42, and 48.75 ± 3.151, respectively (not different, $p > 0.05$). The percentage increase equaled 59.15 ± 13.82, 65.48 ± 15.84, 87.02 ± 19.71, and 81.58 ± 9.644, respectively (not different, $p > 0.05$) when derived from the positive pixel areas. Therefore, there was no change in the coupling efficiency between the receptor and G proteins with steroid treatment.

Western Blot Analysis of $G_{\alpha i3}$, $G_{\alpha i1}$, $G_{\alpha o}$, and $G_{\alpha z}$

Figure 3-8 shows that EE treatment for 30 months significantly reduced $G_{\alpha i3}$ protein levels in the macaque dorsal raphe compared with the OVX controls by unpaired two tailed Student's t test. Levels of $G_{\alpha i1}$, $G_{\alpha o}$, and $G_{\alpha z}$ proteins in EE treated animals were not different from those in the control group.

Hormone Levels

Serum samples were collected from rhesus macaques at necropsy and assayed for E, P, and prolactin. The assays exhibited a less than 9% intra-assay coefficient of variation. The sensitivity of the assays equaled 5 pg/ml for E and 0.1 ng/ml for P and prolactin. The mean (± SEM) concentration of E in the serum of E and E + P treated groups was 105.6 ± 20.02 pg/ml. The mean (± SEM) concentration of P in the serum of P and E + P treated groups was 6.82 ± 1.56 ng/ml. The E level was within the range reported for the mid- to late follicular phase and P level was within the range reported for the mid-luteal phase of the primate menstrual cycle (Hotchkiss, Knobil, 1994). The mean (± SEM) concentrations of E and P in the serum of the untreatd OVX control group were 9.8 ± 4.8 pg/ml and 0.15 ± 0.05 ng/ml, respectively. The mean (± SEM) concentrations of prolactin in the serum of OVX, E, P, and E + P treated groups were 48.53 ± 6.07, 222.55 ± 66.17, 101.60 ± 17.35, and 551.52 ± 120.52, respectively. E + P treatment significantly increased the serum prolactin level compared to OVX control, E, or P treated groups (ANOVA, $p < 0.05$), consistent with previous reports (Williams et al. 1985; Sprangers et al. 1990; Bethea et al. 1992).

DISCUSSION

Results from these experiments demonstrated that one month of E and P significantly decreased 5-HT_{1A} receptor binding sites and G protein activation in macaques. Specifically, postsynaptic 5-HT_{1A} in the hypothalamus was downregulated by E and E + P but not by P alone. The 5-HT_{1A} autoreceptor in the DRN was downregulated by all three hormone treatments. Also in the DRN, the basal and R-(+)-8-OH-DPAT stimulated [^{35}S]GTP-γ-S binding were reduced by each ovarian hormone treatment as well. The expression of $G_{\alpha i3}$ protein, but not of $G_{\alpha i1}$, $G_{\alpha o}$, and $G_{\alpha z}$, in the DRN on Western blots was significantly reduced by conjugated EE.

Postsynaptic 5-HT_{1A} receptor binding in the hypothalamus was down-regulated by E and E + P but not by P alone. The location of 5-HT_{1A} receptor binding was consistent with that of 5-HT_{1A} mRNA expressed in discrete regions in the hypothalamus (Gundlah et al. 1999). However, the steady-state mRNA level of the postsynaptic 5-HT_{1A} in the same regions were not decreased by hormone replacement. Thus, mRNA levels for this receptor do not reflect the functional capacity of the receptor in the postsynaptic target cells. This discrepancy between 5-HT_{1A} mRNA and binding levels may be attributed to the modification of 5-HT_{1A} translational efficiency by hormone treatments. In rats treated with E, postsynaptic 5-HT_{1A} mRNA downregulation occurs days before the downregulation of receptor proteins and then the receptor mRNA level recovers (Osterlund et al. 2000). Alternatively, therefore, the decreased postsynaptic 5-HT_{1A} receptor protein with one month of hormone replacement in monkeys might result from a reduced receptor message somewhat earlier in the treatment.

The downregulation of postsynaptic 5-HT_{1A} in the hypothalamus may affect several aspects of neuroendocrine functions and behaviors. The 5-HT_{1A} receptor is inhibitory and hence, a decrease in receptor availability would remove serotonergic inhibition of the target neurons. For example, 8-OH-DPAT inhibits lordosis and stimulates food intake in rats and E priming before 8-OH-DPAT administration reverses both behavioral effects (Trevino et al. 1999; Jackson, Uphouse, 1996; Salamanca, Uphouse, 1992). In addition, E treatment before 8-OH-DPAT administration also reduces the 5-HT_{1A} mediated release of stress hormones such as corticosterone (Raap et al. 2000).

The binding of [^{3}H]8-OH-DPAT in the DRN reflects the affinity and number of 5-HT_{1A} autoreceptors on the soma and dendrites of serotonin neurons (Verge et al. 1985; Azmitia et al. 1996b). The downregulation of 5-HT_{1A} binding sites in the

monkey raphe by ovarian hormones largely reflected the downregulation of 5-HT_{1A} mRNA observed previously with a similar treatment paradigm (Pecins-Thompson, Bethea, 1999). In contrast to the effect in the hypothalamus, P alone decreased 5-HT_{1A} autoreceptor binding in the DRN. This raises the possibility that P acts by different mechanisms in the hypothalamus and raphe. The region-specific regulation may be due to the phenotype difference between the cells expressing pre- and postsynaptic 5-HT_{1A} receptors. The neurons that express 5-HT_{1A} autoreceptors are serotonergic and contain estrogen receptor β (Gundlah et al. 2000; Gundlah et al. 2001b) and progesterone receptor (Bethea, 1993) whereas serotonin postsynaptic target cells are of numerous phenotypes including GABAergic (Mirkes, Bethea, 2001), glutamatergic (Azmitia et al. 1996b), and oxytocin-producing (Raap et al. 2000). They also express different combinations of estrogen receptor α, β, or progesterone receptor (Bethea et al. 1992; Shughrue et al. 1997; Gundlah et al. 2000). In line with this finding, 5-HT_{1A} agonist mediated receptor desensitization (Kreiss, Lucki, 1997), G protein activation (Sim-Selley et al. 2000), serotonin release (Kreiss, Lucki, 1994), and antidepressant regulation of the 5-HT_{1A} receptor (Chaput et al. 1991) are all brain area specific as well, indicating the characteristic of this receptor is different in different neuronal populations.

5-HT_{1A} receptors are coupled with inhibitory G proteins of $G_{i/o/z}$ families. They negatively regulate adenylate cyclase activity and cell firing (Raymond et al. 1999). This study demonstrated that R-(+)-8-OH-DPAT stimulated [^{35}S]GTP-γ-S binding in the macaque dorsal raphe was reduced during hormone replacement, indicating that ovarian steroids downregulated 5-HT_{1A} autoreceptor mediated G protein activation. Our observation that E and P inhibit the initial step in the 5-HT_{1A} cell signaling is consistent with limited observations in rodents. In rats, E decreases the ability of 8-OH-DPAT to inhibit serotonergic cell firing in the DRN (Lakoski, 1988). The

downregulation of 5-HT_{1A} autoreceptor activity by E and P would disinhibit serotonin release in brain regions containing serotonergic projections. In addition, the basal [^{35}S]GTP-γ-S binding level was also decreased by E, P, or E + P. A likely explanation for this observation is a down-regulation of G protein levels by the hormones.

The generous donation of midbrains from cynomolgus macaques maintained on long-term EE enabled the preliminary examination of steroid regulation of G protein expression. Western blot analysis revealed that the level of $G_{\alpha i3}$ proteins extracted from the dorsal raphe of EE treated macaques was significantly lower than that of OVX controls, but $G_{\alpha i1}$, $G_{\alpha o}$, and $G_{\alpha z}$ did not change. This differs slightly from the observation of Raap et al. (Raap et al. 2000), who found that $G_{\alpha i3}$, $G_{\alpha i1}$, and $G_{\alpha z}$ protein levels are all downregulated by E in the rat hypothalamus. The rat hypothalamus contains a heterogeneous mixture of cell populations that express postsynaptic 5-HT_{1A} receptors, which could couple with a variety of G protein systems. Our observation indicates that in macaque dorsal raphe, $G_{\alpha i3}$ is especially sensitive to steroid modulation. Moreover, $G_{\alpha i3}$ has the highest affinity for the 5-HT_{1A} receptor (Raymond et al. 1999). Therefore, steroid regulation of this G protein subunit could alter the sensitivity of presynaptic 5-HT_{1A} autoreceptor signaling as reflected in our [^{35}S]GTP-γ-S binding results. The long-term treatment of the cynomolgus macaques with EE may limit a direct comparison to the [^{35}S]GTP-γ-S binding data in the rhesus. However, we previously observed a similar increase in tryptophan hydroxylase protein expression in OVX rhesus macaques treated with natural E for a month and OVX cynomolgus macaques treated with EE for 30 months (Bethea et al. 2000).

The mechanisms by which E and P decrease 5-HT_{1A} gene and protein expression are unknown. The steroid effects at the time point investigated in this study were obtained with prolonged treatments and could be attributed largely to the genomic actions of E and P. The promoter region of the 5-HT_{1A} gene contains glucocorticoid

response elements, AP1 sites, and SP1 sites, but no response elements for E or P (Storring et al. 1999; Ou et al. 2000; Meijer et al. 2000; Ou et al. 2001). Transcription factor NF-κ-B stimulates the expression of $5\text{-}HT_{1A}$ gene in CV1-b cells (Meijer et al. 2000). Estrogen receptors β sequester NF-κ-B through protein-protein interactions (An et al. 1999) and thereby repress NF-κ-B driven gene expression (Stein, Yang, 1995). We have found that estrogen receptor β but not α is expressed in macaque serotonin neurons (Gundlah et al. 2000; Gundlah et al. 2001b) and that E treatment decreases the nuclear immuno-detection of NF-κ-B in the DRN (Earl et al. 2001). These data indicate that steroid receptors may inhibit $5\text{-}HT_{1A}$ gene expression via protein-protein interactions with other transcription factors such as NF-κ-B.

Serotonin $5\text{-}HT_{1A}$ receptors, upon activation, undergo protein kinase C and A-mediated receptor phosphorylation and internalization (Raymond et al. 1999). Results from this study indicated that it is unlikely that steroids modified the phosphorylation state of $5\text{-}HT_{1A}$. First, the ligand binding properties of $5\text{-}HT_{1A}$ in all treatment groups were the same as indicated by the similar Kd values between 8-OH-DPAT and the receptor. Second, the coupling efficiency of the receptor with G proteins, which is dependent on the phosphorylation state of the receptor, was not affected by steroids.

In conclusion, one month of hormone replacement down-regulated $5\text{-}HT_{1A}$ postsynaptic receptor binding, $5\text{-}HT_{1A}$ autoreceptor binding, and autoreceptor mediated G protein activation and cell signaling without affecting the intrinsic property of the receptors in non-human primates. These data suggest that hormone replacement could be a beneficial adjunct to antidepressant treatment in postmenopausal women with mood disorders by decreasing the $5\text{-}HT_{1A}$ autoreceptor-specific second message pathway. It is also important to note that in this study, the rhesus macaques were treated with natural hormones, which differ in many respects from synthetic compounds commonly prescribed for hormone replacement therapy. For women at

risk for breast and uterine cancer, however, hormone replacement therapy is not an option. Hence, the development of a selective estrogen receptor modulator with efficacy in the brain serotonin system but without any detrimental effects in the peripheral tissues is needed.

Post-synaptic 5-HT_{1A} in the Hypothalamus

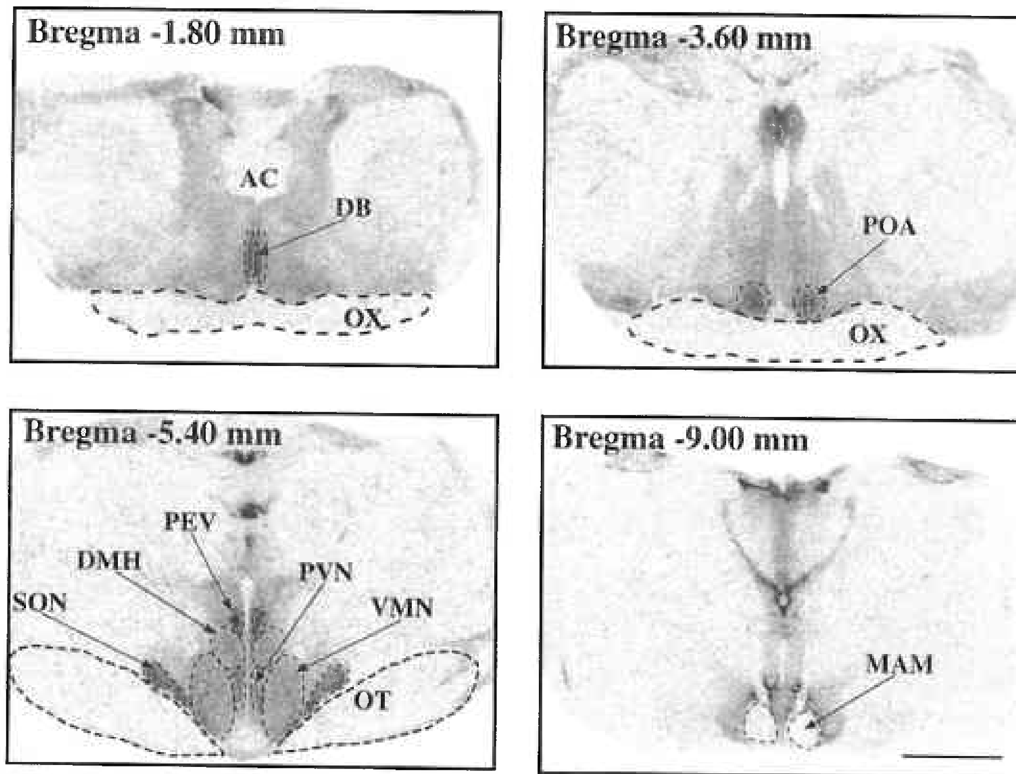


Figure 3-1. Autoradiograms of [^{3}H]8-OH-DPAT binding on coronal monkey hypothalamic sections (10 μm) in a rostral to caudal direction. The dark areas are autoradiographic signals representing 5-HT_{1A} receptor density in different hypothalamic nuclei. Significant signal intensity was detected in the vertical limb of the diagonal band of Brocca (DB), preoptic area (POA), supraoptic (SON), paraventricular (PVN), periventricular (PEV), ventromedial nucleus (VMN), and dorsal medial hypothalamus (DMH). For anatomical orientation, the following landmarks are indicated. AC (anterior commissure), MAM (mammilary body), OT (optic tract), and OX (optic chiasm). Scale bar = 5 mm.

Ovarian steroid regulation of $[^3H]$8-OH-DPAT binding in the hypothalamus

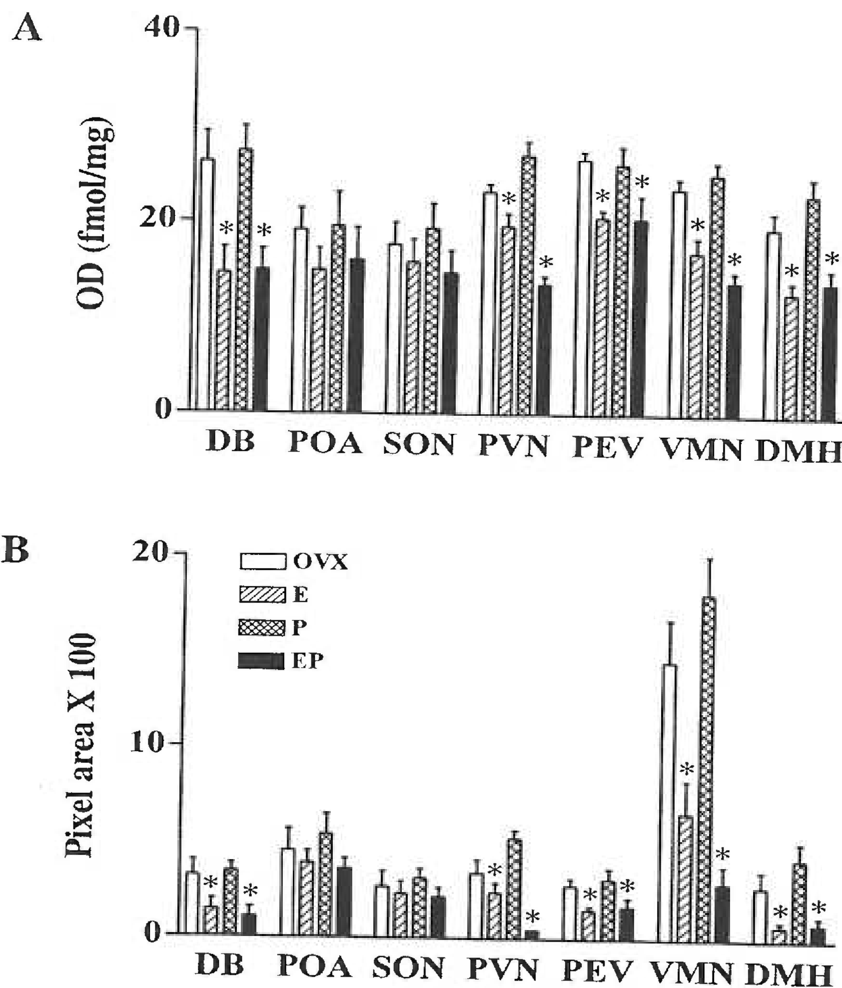


Figure 3-2. Comparison of $[^3H]$8-OH-DPAT binding sites in the monkey hypothalamic sections from OVX, E (28 d), P (28 d), and E (28 d) plus P (last 14 d) treated macaques. A. Average optical density values from four to six levels of each hypothalamic nucleus (n = 5/treatment). Compared to the OVX control animals using ANOVA, 5-HT_{1A} binding sites were significantly decreased by E, or E + P but not by P alone in the DB ($p = 0.004$, F = 6.9, df = 18), PVN ($p < 0.0001$, F = 24, df =18), PEV ($p = 0.0254$, F = 4.1, df =18), VMN ($p < 0.0001$, F =19, df =18), and DMH ($p = 0.0005$, F =11, df =18). B. Positive pixel areas reflecting the $[^3H]$8-OH-DPAT binding in the hypothalamus. Compared to the OVX control animals, $[^3H]$8-OH-DPAT labeled positive pixel areas were significantly decreased by E, or E + P but not by P alone in the DB ($p = 0.0155$, F = 4.8, df =18), PVN ($p < 0.0001$, F = 20, df =18), PEV ($p = 0.042$, F = 3.5, df =18), VMN ($p < 0.0001$, F =17, df =18), and DMH ($p = 0.0025$, F = 7.6, df =18). $[^3H]$8-OH-DPAT binding sites were not changed by any of the treatments in the POA or SON. * significantly different from the OVX control group.

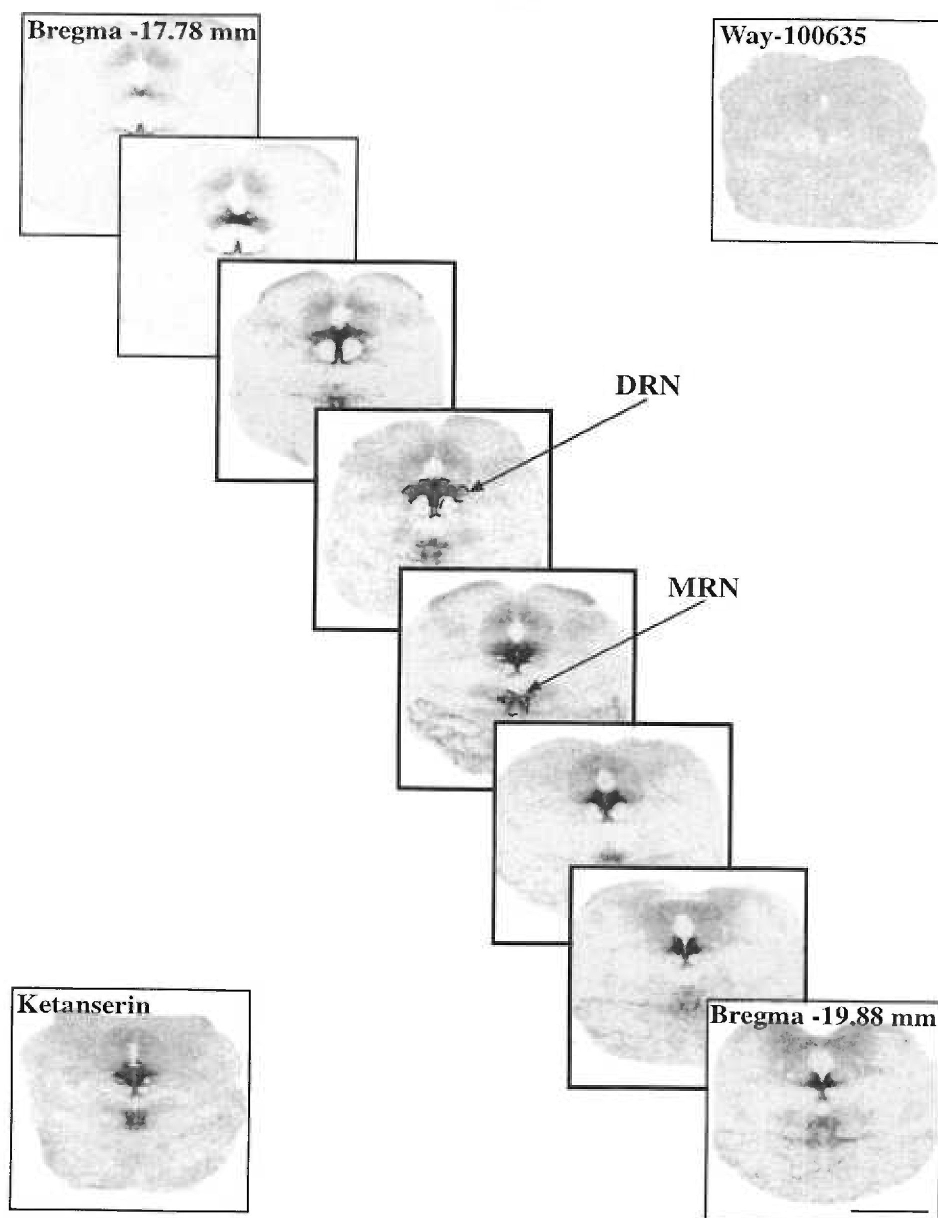
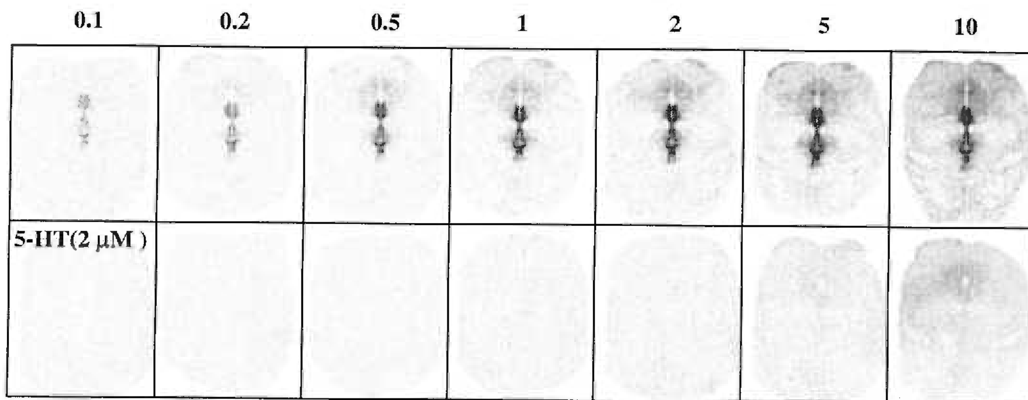


Figure 3-3. Autoradiograms of [^{3}H]8-OH-DPAT binding on monkey midbrain sections (10 µm). Midbrain sections through eight levels of the DRN with an interval of 300 µm are shown. The five levels yielding the average numbers of 5-HT_{1A} binding sites are in dark frames. [^{3}H]8-OH-DPAT binding was dense in the DRN, moderate in the median raphe nucleus (MRN) and light in the periaquiductal gray. Way100635 (1 µM) effectively blocked 5-HT_{1A} labeling. Ketanserin (1 µM) did not affect 5-HT_{1A} labeling. Scale bar = 5 mm.



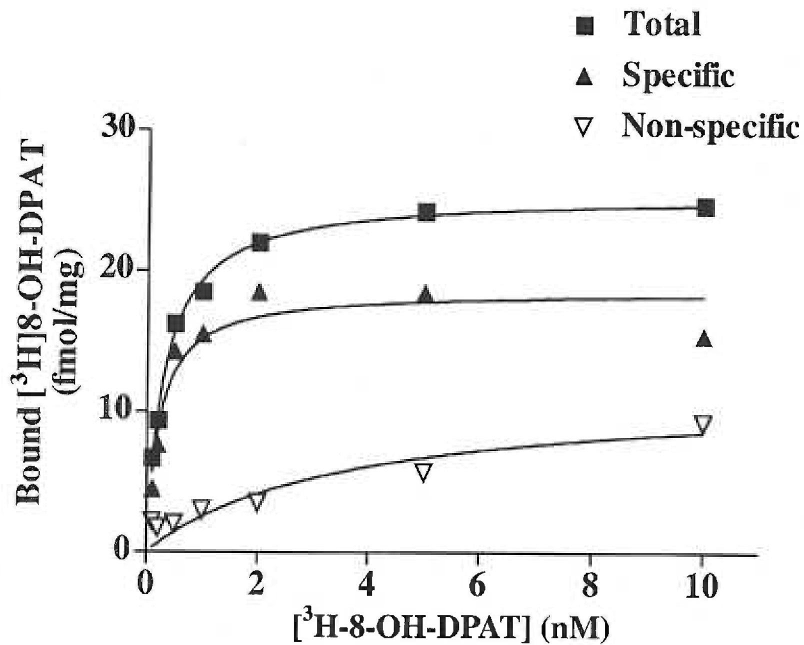


Figure 3-4. Saturation studies of [^{3}H]8-OH-DPAT binding in the DRN on monkey midbrain sections (10 μm). Serial sections were incubated with seven concentrations of [^{3}H]8-OH-DPAT ranging from 0.1 nM to 10 nM. Densitometric analysis of [^{3}H]8-OH-DPAT binding sites was performed with NIH Image software. One site curve fitting was performed with Prism 3.0. [^{3}H]8-OH-DPAT binding in the monkey DRN reaches equilibrium at 1 to 2 nM. The non-specific binding was below 15% of the total binding.

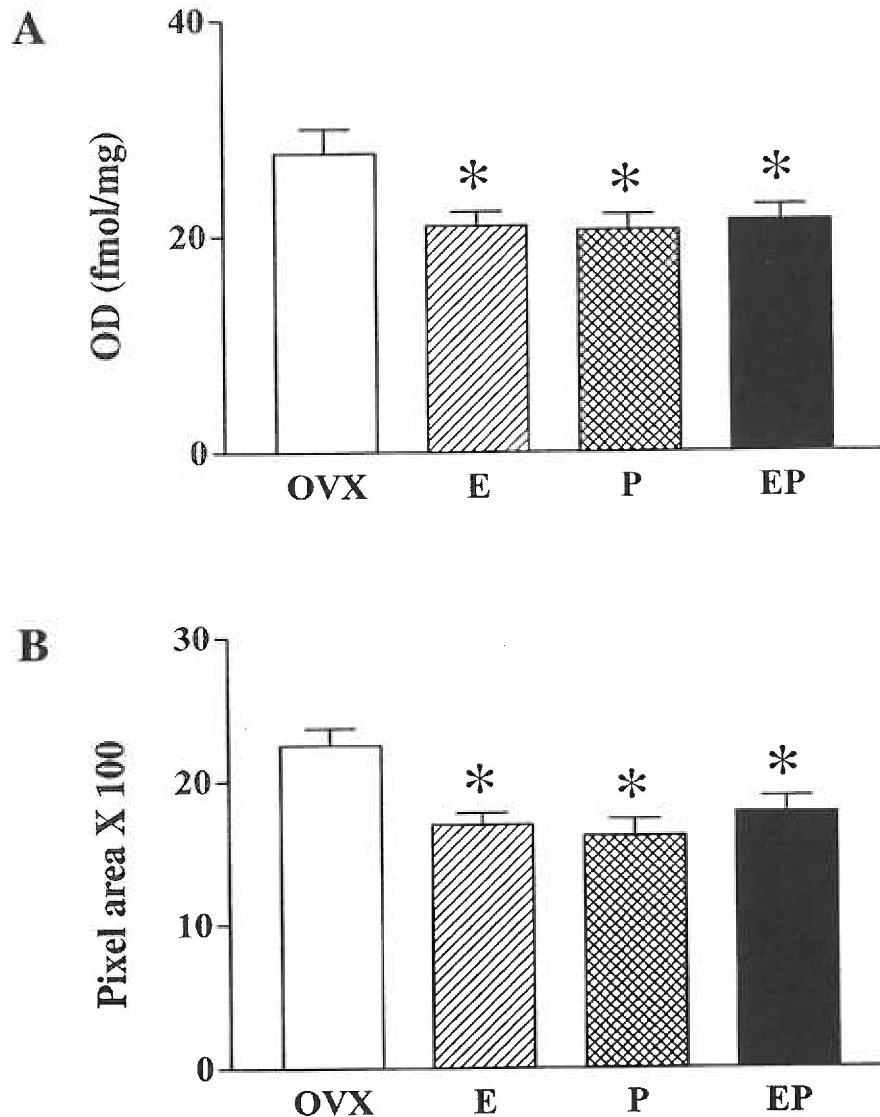


Figure 3-5. Comparison of [^{3}H]8-OH-DPAT binding levels in the monkey DRN from OVX, E (28 d), P (28 d), and E (28 d) plus P (last 14 d) treated macaques. A. Average optical density values from five levels of the DRN (n = 5/treatment). Compared to the OVX control animals, 5-HT$_{1A}$ binding sites were significantly decreased by E, P, and E + P (ANOVA, $p = 0.027$, F = 4.1, df =18). B. Positive pixel areas reflecting [^{3}H]8-OH-DPAT binding in the DRN. Compared to the OVX control animals, [^{3}H]8-OH-DPAT generated pixel areas in the DRN were significantly decreased by all three treatments (ANOVA, $p = 0.006$, F = 6.2, df = 18). * significantly different from the OVX control group.

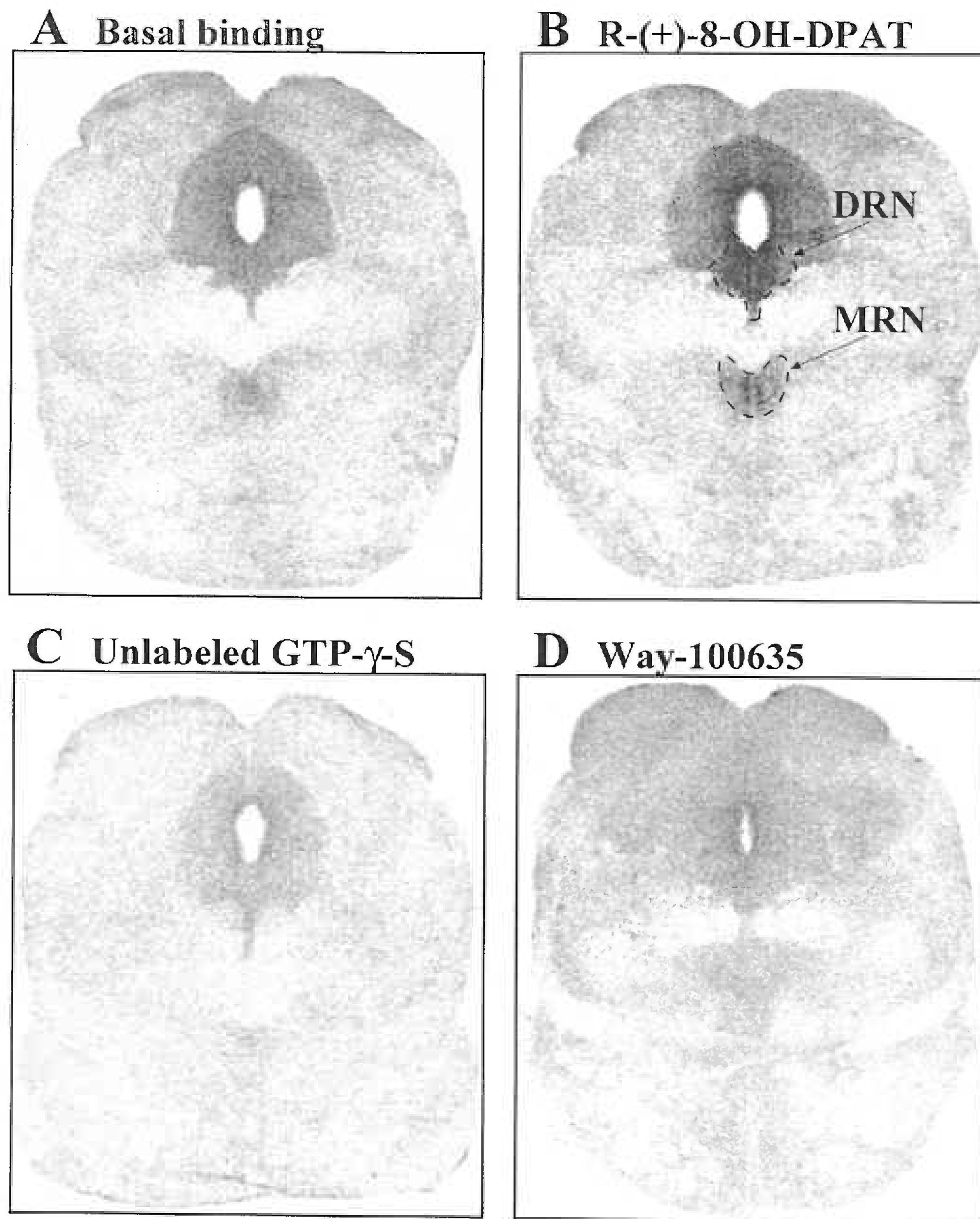


Figure 3-6. Autoradiograms of [^{35}S]GTP-γ-S labeling in the monkey midbrain sections (10 μm). A. The basal binding of [^{35}S]GTP-γ-S was diffuse. B. The stimulated binding of [^{35}S]GTP-γ-S in the presence of 1 μM of R-(+)-8-OH-DPAT was robust in the DRN and moderate in the MRN and the periaquiductal gray. C. Non-specific binding was defined in the presence of 10 μM unlabeled GTP-γ-S. D. The R-(+)-8-OH-DPAT stimulated [^{35}S]GTP-γ-S binding in the DRN can be blocked by the selective 5-HT_{1A} receptor antagonist Way100635 (1 μM) but not by phentolamine (data not shown). Scale bar = 5 mm.

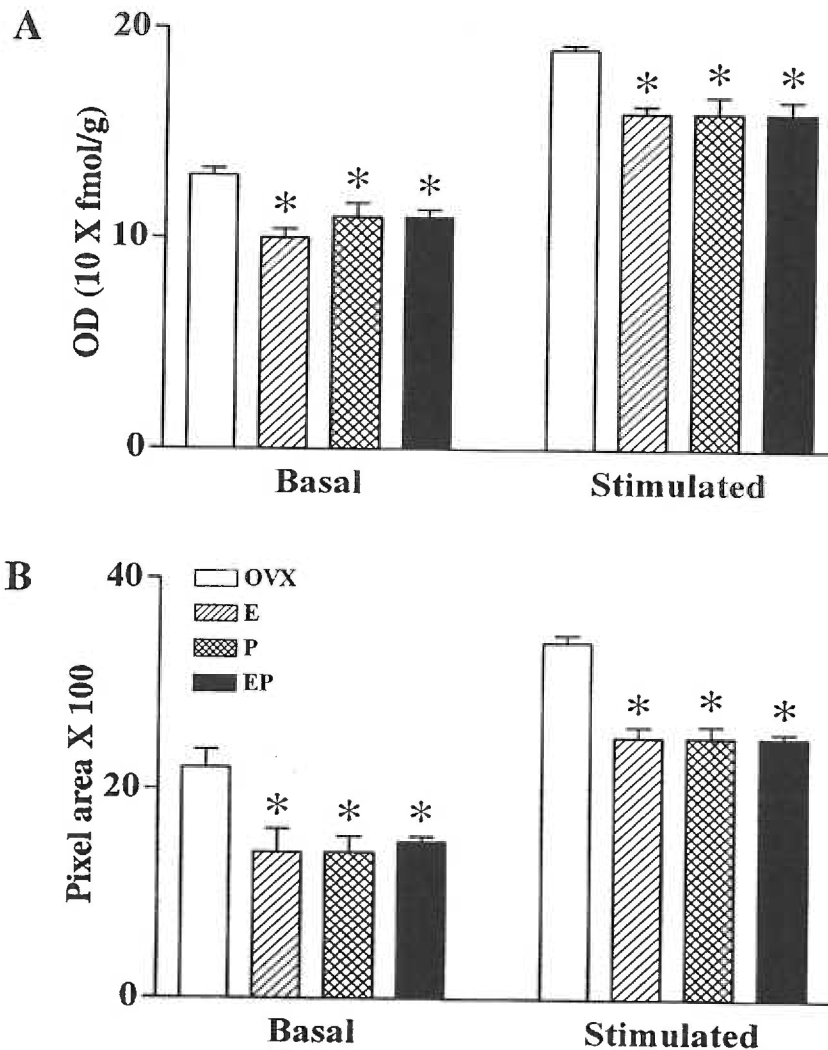


Figure 3-7. Average number of [^{35}S]GTP-γ-S binding sites in the midbrain sections from OVX, E (28 d), P (28 d), and E (28 d) plus P (last 14 d) treated macaques. A. Average optical density values from five levels of the DRN (n = 5/treatment). Compared to the OVX control animals with ANOVA, basal [^{35}S]GTP-γ-S binding sites were significantly decreased by E, P, or E + P ($p = 0.0087$, F = 5.8, df =17). R-(+)-8-OH-DPAT stimulated [^{35}S]GTP-γ-S binding sites in all treatment groups were also decreased ($p = 0.0011$, F = 9.6, df =17). B. Pixel areas reflected the optical density values for both the basal and stimulated [^{35}S]GTP-γ-S binding in the DRN. Compared to the OVX control animals, positive pixel areas representing the basal [^{35}S]GTP-γ-S labeling were significantly decreased by E, P, or E + P ($p = 0.0135$, F = 5.1, df = 17). The positive pixel areas representing the stimulated binding were also decreased in all treatment groups ($p < 0.0001$, F = 23, df =17). * significantly different from the OVX control group.

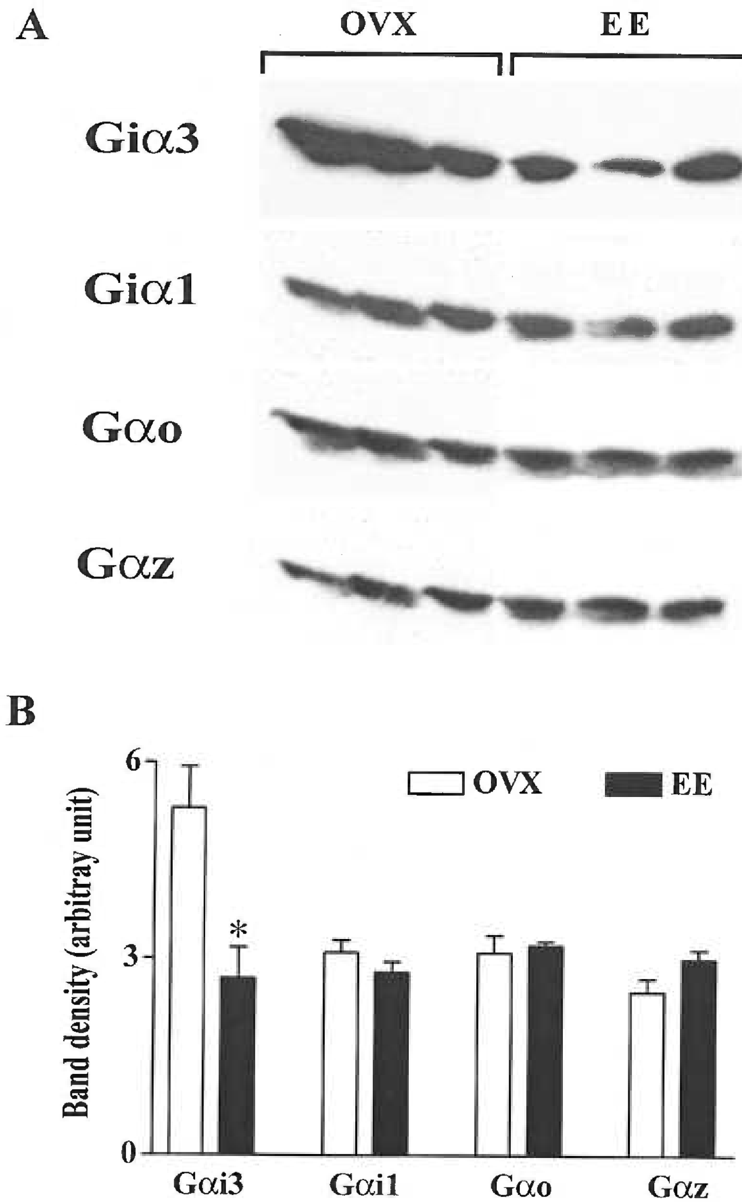


Figure 3-8. Equine estrogen (EE, 30 months) significantly reduced $G_{\alpha i3}$ protein levels. A. G protein subunit signal bands detected on Western blots. B. Densitometric analysis showed a significant decrease of the $G_{\alpha i3}$ band density by EE treatment compared to the control group with unpaired two tailed t test ($p = 0.0297$, $t = 3.3$, $df = 3$). The levels of $G_{\alpha i1}$, $G_{\alpha o}$, and $G_{\alpha z}$ proteins were not different between EE treated and OVX control groups. * significantly different from the OVX control group.

CHAPTER 4 Ovarian Steroid Regulation of Serotonin Reuptake Transporter (SERT) Binding and Serotonin Uptake in Adult Macaques

ABSTRACT

The serotonin reuptake transporter (SERT) plays an important role in serotonin neurotransmission. We previously demonstrated that estradiol (E) with or without progesterone (P) decreased SERT mRNA levels in the macaque dorsal raphe nucleus (DRN). In this study, we investigated whether E and P regulate SERT binding, protein expression, and function. Quantitative autoradiography for SERT using [^{3}H]citalopram was performed on brain sections of rhesus macaques from four treatment groups: ovariectomized controls (OVX), E (28 d), P (28 d), and E (28 d) plus P (the last 14 d). Western blot analysis for SERT was performed on DRN extracts from cynomolgus macaques that were OVX or treated with equine estrogens (EE, 30 months). [^{3}H]Serotonin uptake was measured in forebrain synaptosomal preparations of the rhesus macaques. In the raphe, one month of E, P, and E+P treatments did not change SERT binding. However, 30 months of EE treatment increased SERT protein expression on Western blots. In several hypothalamic nuclei, [^{3}H]citalopram binding levels were increased by E, P, and E+P. Finally, E and E+P treatment increased serotonin uptake in another serotonergic target, the basal ganglia without changing the Km values. These observations suggest that SERT binding sites and protein expression in the raphe do not reflect SERT mRNA expression during hormone replacement. In addition, SERT binding sites and activity were upregulated in selected serotonergic terminals. The steroid-induced increase in SERT binding and activity in serotonin terminal fields supports the notion that ovarian hormones increase serotonin neurotransmission and that synaptic serotonin may prevent SERT degradation.

Serotonin plays a critical role in several autonomic brain functions including integrated cognition, sleep, mood, satiety, hormone secretion, and sexual behaviors (van de Kar, 1991; Jacobs, Azmitia, 1992). The primary means by which serotonergic neurons terminate extracellular serotonin signaling is via the serotonin reuptake transporter (SERT), which is predominantly located pre-synaptically at serotonin nerve terminals with some expression on serotonin cell bodies and dendrites (Lesch et al. 1993; Blakely et al. 1994). SERT protein levels in the midbrain of patients with major depression are reduced, compared to those of healthy individuals (Malison et al. 1998; Gross-Isseroff et al. 1998). Furthermore, blunted serotonin release and uptake has been associated with depression and other psychiatric disorders (Rausch et al. 1982; Faludi et al. 1988; Mann et al. 1992; Halbreich, Tworek, 1993; Cleare, 1997).

A body of evidence suggests that ovarian hormones can impact mood and cognition (McEwen, 1999). The identification of estrogen receptor β and progesterone receptor in serotonin neurons of primates suggests that the beneficial psychological effects of estrogen (E) and progesterone (P) could be transduced by serotonin neurons (Gundlah et al. 2001b; Bethea, 1993). We previously demonstrated that E with or without P decreased (SERT) mRNA in the dorsal raphe nucleus (DRN) of ovariectomized (OVX) macaques (Pecins-Thompson et al. 1998). This observation led to the speculation that a decrease in SERT gene expression may cause a decrease in SERT protein expression, which, in turn, could elevate serotonin concentration in the synapse and overall serotonin neurotransmission. However, several pieces of evidence are not consistent with this line of reasoning. In a clinical study, platelet SERT binding sites were significantly increased after E treatment in surgically induced menopausal women (Sherwin, Suranyi-Cadotte, 1990). Furthermore, SPECT studies and a study using postmortem tissue indicate that persons with depression have lower serotonin reuptake sites than healthy controls (Malison et al. 1998; Gross-Isseroff et al. 1998).

In addition, the majority of the studies on rodents indicate that E increases SERT gene and protein expression in the brain (Ravizza et al. 1985; Rehavi et al. 1987; Attali et al. 1997; McQueen et al. 1997; McQueen et al. 1999; Sumner et al. 1999). Moreover, in HEK-293 cells expressing SERT, when there is more serotonin available, there is also more uptake (Ramamoorthy, Blakely, 1999; Bauman et al. 2000). Therefore, in general, there is a positive correlation between the level of SERT protein and the amount of synaptic serotonin.

In this study, quantitative autoradiography was used to measure and compare SERT binding sites in the raphe and in the serotonergic target sites in the hypothalamus of hormone treated macaques and placebo-treated OVX controls. SERT protein expression was also measured with Western blot analysis in dorsal raphe extracts from OVX macaques treated with placebo or conjugated equine estrogen (EE). In addition, the effect of E and P on SERT function in serotonergic terminal fields, specifically the basal ganglia and the hippocampus, was examined by determining the level of serotonin uptake in these regions.

SPECIFIC METHODS

[^{3}H]Citalopram Binding

SERT binding experiments were performed according to Duncan et al. (Duncan et al. 1992) with minor modifications. Briefly, slides were brought to room temperature in a vacuum and preincubated with assay buffer (50 mM Tris, 120 mM NaCl, and 5 mM KCl, pH 7.4) at 22C for 15 min. Slides were then incubated with 2 nM [^{3}H]citalopram in the assay buffer at 22C for 1 hr. Non-specific binding was defined with the presence of 1 μM fluoxetine on adjacent sections. Citalopram, mazindol, GBR12935 at 0.1 and 1 μM were also added in some experiments to evaluate the selectivity of the assay. After incubation, the slides were washed in the

assay buffer for 1 min followed by washing in the assay buffer twice for 10 min each and rinsing in distilled water for 1 min at 4C. The sections were dried rapidly under a stream of cold air.

Matching sections from OVX controls, E treated, P treated and E+P treated monkeys were processed in the same experiment and exposed to ^{3}H-sensitive hyperfilms along with ^{3}H autoradiographic micro-scales (Amersham, Arlington Heights, IL) for three to five weeks. Autoradiograms were digitized with a SONY CCD video camera. Densitometry was performed using NIH image on a Macintosh computer. Each film was calibrated with ^{3}H autoradiographic micro-scales.

Western Blot Analysis for SERT

Western blotting was performed according to the modified procedures of Qian et al. (Qian et al. 1995) with blotting buffer containing 25 mM of Tris base and 192 mM of glycine. The nitrocellulose membranes (Osmonics, Westborough, MA) were blocked in 5% non-fat dry milk for 45 min before incubating with primary antibodies at 4C overnight. The dilutions for anti-SERT and $G_{\alpha i1}$ were 1:600 and 1:150, respectively. The following morning, the blots were washed in saline and 0.05% tween-20 (Bio-Rad) and incubated with secondary antibody conjugated to HRP at 1:2000 at room temperature for 2 hr and then developed with Supersignal chemiluminesence kits (Pierce, Rockford, IL) followed by exposure to Kodak X-OMAT AR film. Densitometric analysis of signal bands was performed using NIH Image Gel Plotting software.

In addition, to assess the regulation of SERT by serotonin *in vitro*, the same amount of midbrain extract was incubated with or without added serotonin (0.4 nmol/μg total protein) for 30 to 120 min in the absence or presence of protease

inhibitors listed above before processing for Western blot analysis. Citalopram (0.4 nmol/μg) was used to block the effect of serotonin.

[^{3}H]Serotonin Uptake in Monkey Brain Synaptosomes

Monkey frontal cortices, temporal, and parietal lobes were thawed in 0.32 M sucrose at room temperature and microdissected to obtain area 46, cingulate, striata, and hippocampi. The tissues were weighed and homogenized in 10 volumes of 0.32 M sucrose at 4C by hand with 10 to 20 strokes of a glass-Teflon homogenizer. The homogenate was centrifuged at 1000 X g for 10 min at 4C, and the supernatant was further centrifuged at 20,000 X g for 20 min at 4C. The resulting pellet was resuspended in 7 volumes (original wet weight of tissue) of 0.32 M glucose at 4C. Synaptosomal preparations (50 μl, 100 to 200 μg protein) were preincubated in Krebs-HEPES buffer (25 mM HEPES, 122 mM NaCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, pH 7.4) supplemented with 10 μM pargyline, 100 μM tropolone, 30 nM methiothepin, 0.2% glucose, and 0.02% ascorbic acid in 1-ml assay tubes (Marsh Biomedical, Rochester, NY) at 30C for 10 min. [^{3}H]Serotonin uptake was initiated by the addition of serotonin at eight concentrations ranging from 10 to 810 nM and terminated after 10 min by filtration through Wallac A filters presoaked in 0.05% polyethylenimine using a Tomtec 96-well cell harvester. Serotonin at each concentration contained 10 nM of [^{3}H]serotonin and 0 to 800 nM of unlabeled serotonin. Specific uptake was defined as the difference in uptake observed in the absence and presence of 5 μM fluoxetine. The final assay volume in each tube was 500 μl. Filters were washed for six seconds with saline and air-dried. Scintillation fluid (β-Scint) was added to each filtered spot and radioactivity remaining on the filters was determined using a Wallac β-plate reader. Each experiment was conducted with triplicate determinations. Protein was determined with a modified method of Lowry et al. (Lowry et al. 1951).

In addition, selectivity of [^{3}H]serotonin (10 nM) uptake by monkey striatal synaptosomes was determined in the presence of citalopram, fluoxetine, mazindol, and GBR12935 at concentrations ranging from 0.1 nM to 10 μM. The first two drugs are selective serotonin reuptake inhibitors (SSRIs) and have high affinity for SERT. In contrast, mazindol has high affinity for norepinephrine transporter whereas GBR12935 has high affinity for dopamine transporters (Eshleman et al. 1999).

RESULTS

Distribution and Regulation of [^{3}H]Citalopram Binding in Monkey Raphe

[^{3}H]Citalopram labeling on monkey midbrain sections reflected the distribution pattern of SERT mRNA (Pecins-Thompson et al. 1998), i.e., robust in the dorsal raphe, moderate in the median raphe, and light in the periaquiductal gray (Figure 4-1). Non-specific SERT binding defined by the presence of 1 μM fluoxetine was below 15% of the total [^{3}H]citalopram binding. In addition, 100 nM of fluoxetine, but not mazindol and GBR12935, blocked [^{3}H]citalopram binding.

E, P, and E+P treatments did not change the density of SERT binding sites in the DRN and median raphe nucleus (MRN) compared to the OVX controls (Figure 4-2). The average OD values from six levels of the DRN and MRN (n=5/treatment) were not different between treatment groups. Positive pixel areas, which relate well with the number of cells with positive SERT labeling, reflected the OD values. Compared to the OVX control animals, the positive pixel area representing [^{3}H]citalopram binding in the DRN and MRN were not changed by any of the treatments.

Western Blot Analysis of SERT

Figure 4-3 shows that EE treatment for 30 months significantly increased SERT protein (78 kd) levels in the macaque dorsal raphe compared with the OVX controls by unpaired two tailed Student's t test. We previously found that the mass of $G_{i\alpha 1}$ in the raphe was not altered by hormone treatments (Lu, Bethea, 2001). Therefore, Western blots were probed for $G_{i\alpha 1}$ band density to normalize for sample loading.

In addition, as shown in figure 4-4, SERT degradation was inhibited by the addition of serotonin (0.4 nmol/μg total protein) *in vitro*. The protective effect of serotonin was blocked by citalopram (0.4 nmol/μg).

Distribution and Regulation of [^{3}H]Citalopram Binding in the Monkey Hypothalamus

SERT binding sites in the serotonergic projection fields in the hypothalamus are widespread. A discrete pattern of [^{3}H]citalopram binding in the hypothalamus was observed in the anterior hypothalamus (AH), lateral tuberal nuclei (LTu), periventricular nuclei (PeV), VMN, lateral hypothalamus (LH), dorsal medial hypothalamus (DMH), and mammillary bodies (MAM) (Figure 4-5).

The average of positive pixels and OD values from four to six levels of each hypothalamic region (n=5/treatment) were compared by ANOVA. Compared to the OVX control animals, SERT binding sites represented by positive pixel areas were significantly increased by E, P, or E+P in the AH, VMN, LH, and DMH (Figure 4-6). OD values representing SERT binding sites generally reflected the pixel areas in each hypothalamic region examined. In LTu, PeV, and MAM, neither the OD nor the positive pixel area of [^{3}H]citalopram binding was different among treatment groups.

[^{3}H]Serotonin Uptake

Km values for specific serotonin uptake measured in monkey synaptosomes prepared from cryopreserved and fresh raphes were not different and the Vmax of cryopreserved tissue reached about half of the Vmax value obtained from fresh tissue (Figure 4-7). Subsequently, the Km and Vmax values were examined in multiple brain regions of the same twenty animals used for [^{3}H]citalopram binding experiments. The basal ganglia and hippocampi had robust serotonin uptake whereas the uptake in area 46, cingulate gyrus, occipital lobe of the cortex and the thalamus were below the assay sensitivity.

The IC_{50} (± SEM) values for citalopram, fluoxetine, mazindol, and GBR12935 at inhibiting serotonin uptake in basal ganglia were 0.46 ± 0.03 nM, 2.85 ± 0.18 nM, 150 ± 10 nM, and 18.13 ± 8.82 μM, respectively, indicating the assay measured the transport through SERT selectively (Figure 4-8. A).

Specific serotonin uptake saturated at 150 to 350 nM serotonin in all treatment groups (Figure 4-8. B). E and E+P treatment significantly increased the Vmax of specific serotonin uptake in the basal ganglia compared to the OVX controls (two-way ANOVA). Serotonin uptake in the hippocampus was not changed by any of the treatments (Table 4-1). The Km values were not different among treatment groups in these two serotonergic terminal fields (two-way ANOVA).

DISCUSSION

A body of evidence suggests that ovarian hormones can impact mood or cognition via the serotonin neural system (McEwen, 1999; Mann, 1999) and SERT is thought to be a pivotal regulation point for serotonin neurotransmission (Blakely et al. 1994). The SSRIs, a class of effective antidepressants, act at SERT and block the transport of serotonin through the transporter. Questions regarding the efficacy of SSRIs in postmenopausal women highlight the importance of understanding how

ovarian hormones impact the functions of SERT. In this study, we determined the effects of ovarian hormones on brain SERT binding, protein expression, and SERT activity in macaques.

At somatodendritic locations in the raphe, binding sites and protein expression of SERT did not reflect SERT mRNA levels in hormone treated macaques as previously documented (Pecins-Thompson et al. 1998). E and E+P significantly decreased the level of SERT mRNA in the DRN of macaques. However, a similar treatment regimen did not change the density of SERT binding sites in the same area. Moreover, thirty months of EE treatment significantly increased SERT protein expression in Western blot analyses. Furthermore, in this study, we demonstrated that the degradation of SERT protein was inhibited by serotonin *in vitro*.

At hypothalamic serotonergic nerve terminals, E, P, and E+P increased the density of SERT binding sites in several nuclei including the AH, VMN, LH, and DMH. E and E+P also increased serotonin uptake at another serotonergic projection site, the basal ganglia, but not at the hippocampus. The affinity of SERT for serotonin was not changed by steroid treatments.

Thus, we have observed a discrepancy between SERT mRNA and protein expression, as well as a region specific regulation of SERT binding. These findings give credence to the notion that the protein expression and activity of SERT are regulated by the level of extracellular serotonin (Ramamoorthy, Blakely, 1999). Functional SERT proteins on the cell membrane exist in a dynamic multimeric complex with phosphatase 2A and the stability of this complex is usage dependent (Bauman et al. 2000). When the level of extracellular serotonin is high, SERT will continuously pump serotonin to the inside of cells. The translocation of serotonin through the portal of SERT strengthens the interaction between SERT and phosphatase 2A, thus preventing SERT phosphorylation and SERT internalization. According to this model,

the level of functional SERT membrane protein should positively correlate with serotonin levels in the extracellular space.

Clinical data and findings of animal studies are consistent with this theory and our data. In depressed patients serotonin and SERT levels are both low compared to healthy individuals (Rausch et al. 1982; Faludi et al. 1988; Mann et al. 1992; Halbreich, Tworek, 1993; Cleare, 1997; Malison et al. 1998; Gross-Isseroff et al. 1998). In SERT knock-out mice, brain serotonin concentrations are also reduced 60-80% (Bengel et al. 1998). In rats, E treatment increases the level of both SERT binding and steady-state mRNA in the DRN (McQueen et al. 1997; McQueen et al. 1999; Sumner et al. 1999).

We propose that the hormone replacement regimen employed in this study stimulates serotonin neurotransmission by altering several crucial components in serotonin neurons. First, E and P increase the gene and protein expression of tryptophan hydroxylase (TPH), the committal enzyme for serotonin synthesis, in female monkeys (Pecins-Thompson et al. 1996; Bethea et al. 2000), suggesting an increase in serotonin production. Second, E and P decreased the expression and activity of the 5-HT_{1A} autoreceptor in macaques, indicating that ovarian steroids may increase serotonin neuronal firing and serotonin release (Lu, Bethea, 2001). Third, the expression of monoamine oxidase-A, a serotonin metabolic enzyme was decreased by similar treatments in the monkey DRN, which may result in a decrease of serotonin degradation (Gundlah et al. 2001a). All of these actions may synergistically elevate extracellular serotonin, thereby preventing SERT internalization and degradation. When SERT proteins are being preserved, serotonin neurons may decrease the expression of SERT mRNA although the feedback mechanism is unknown.

SERT binding sites in the raphe and at serotonin nerve terminals were regulated differentially by ovarian hormones. One month of E, P, or E+P treatment did not change the density of SERT binding sites in the raphe of macaques compared to the

OVX controls. On the other hand, all three hormone treatments increased the density of SERT binding sites and uptake at some serotonergic terminal fields. These data suggest that the regulation of functional SERT binding sites is specific for different brain regions and raises the issue of SERT protein sorting and trafficking. The machinery that manufactures SERT mRNA and protein is mainly in the serotonin cell soma. SERT assembled in the serotonin cell body is then modified while being trafficked to the serotonin cell membrane at the dendrites, cell body, and nerve terminals. Electron microscopy studies indicate that SERT proteins distribute within serotonin neurons in a polarized fashion (Tao-Cheng, Zhou, 1999). That is, SERT labeling is dense on the plasma membrane along the axons and peri-synaptic areas and concentrated in the cytoplasm of the soma and dendrites. E and P have been found to modify microtubule dynamics (Karbowski et al. 2001) and further studies are needed to better understand the factors determining SERT protein sorting and trafficking.

Although SERT binding sites in the raphe were not changed by one month of E and P, SERT protein expression as measured by Western blot analysis was significantly higher in the raphe extracts from monkeys treated with EE for 30 months compared to OVX controls. It is possible that the difference we observed between SERT binding sites and SERT protein expression during hormone replacement is due to the difference in the duration of the treatments. Alternatively, SERT tertiary conformation or trafficking may be affected by steroid treatment. Electron microscopy of immunolabeling using an anti-SERT antibody raised against the first 71 amino acids of the SERT N-terminus has revealed that SERT in the somatodendritic raphe region is concentrated on the membranes of rough endoplasmic reticulum and Golgi complexes but not on the cell surface (Tao-Cheng, Zhou, 1999). This suggests that a large portion of SERT in the raphe region is newly synthesized or undergoing post-translational modifications. The anti-human SERT ST52-1 antibody we used for Western blot

analysis was raised against the N-terminus of SERT as well. However, it is not known whether the ligand-binding to the SERT in the cytoplasm of serotonin neurons was compromised as compared to the binding to the SERT expressed on the cell membrane. Thus, Western blot analysis may detect SERT that is not in a ligand binding conformation.

Limited clinical data indicate that ovarian steroids may facilitate the therapeutic effects of SSRIs. In the presence of E, SSRI antidepressants are more efficacious than in the absence of the hormone (Schneider et al. 1997) though this report was limited by a number of factors and no large, prospective, controlled studies addressing this issue have been reported. Nonetheless, m-CPP, a serotonergic agonist, in a challenge test paradigm, is more potent in the presence of E (Halbreich et al. 1995). In addition, prolonged E replacement increases central 5-HT tone in healthy postmenopausal women (van Amelsvoort et al. 2001). The mechanisms by which SSRIs achieve therapeutic efficacy are not well understood and whether SSRIs regulate SERT gene expression is controversial (Lesch et al. 1993; Spurlock et al. 1994; Neumaier et al. 1996; Benmansour et al. 1999). The majority of studies report no effect of chronic SSRI treatment on SERT binding sites (Graham et al. 1987; Cheetham et al. 1993; Gobbi et al. 1997). Thus, the actions of ovarian hormones and SSRIs seem to converge upon the concentration of serotonin in the extracellular space, whereby each acts by different mechanisms to increase the level of the transmitter in the synapse. This notion predicts that what has been observed as a possible increase in the efficacy of SSRIs by hormone replacement in women may, in stead, be an additive effect of ovarian hormones and an SSRI.

In conclusion, hormone replacement in non-human primates did not change SERT binding sites in the raphe, but increased SERT protein expression at somatodendritic locations and increased the density of SERT binding and serotonin

uptake in selected serotonergic terminal fields without affecting the affinity of the transporter. These changes at the level of SERT protein and function do not follow from changes in SERT gene expression. Rather, the up-regulation of SERT binding and uptake in selected brain areas may have resulted from the elevated extracellular serotonin in these regions. Hence, ovarian hormones may stimulate various aspects of serotonin neurotransmission in a coordinated fashion.

Table 4-1 Vmax (pmol/mg/10 min ± SEM) and Km (μM ± SEM) of specific [^{3}H]5-HT uptake in monkey synaptosomes.

	Vmax		Km	
	Basal Ganglia	**Hippocampus**	**Basal Ganglia**	**Hippocampus**
OVX	3.82 ± 0.43	4.97 ± 0.78	0.09 ± 0.02	0.20 ± 0.08
E	8.04 ± 1.29*	6.59 ± 1.44	0.21 ± 0.08	0.32 ± 0.13
P	5.62 ± 0.77	6.13 ± 1.62	0.14 ± 0.05	0.43 ± 0.20
E+P	9.60 ± 1.67*	4.84 ± 0.88	0.17 ± 0.08	0.30 ± 0.12

*** Significantly different from OVX controls (Two-way ANOVA with OVX, E, and E+P; Newman-Keuls post-hoc pairwise comparison, p = 0.0445).**

[^{3}H]Citalopram Binding in the Midbrain of Macaques

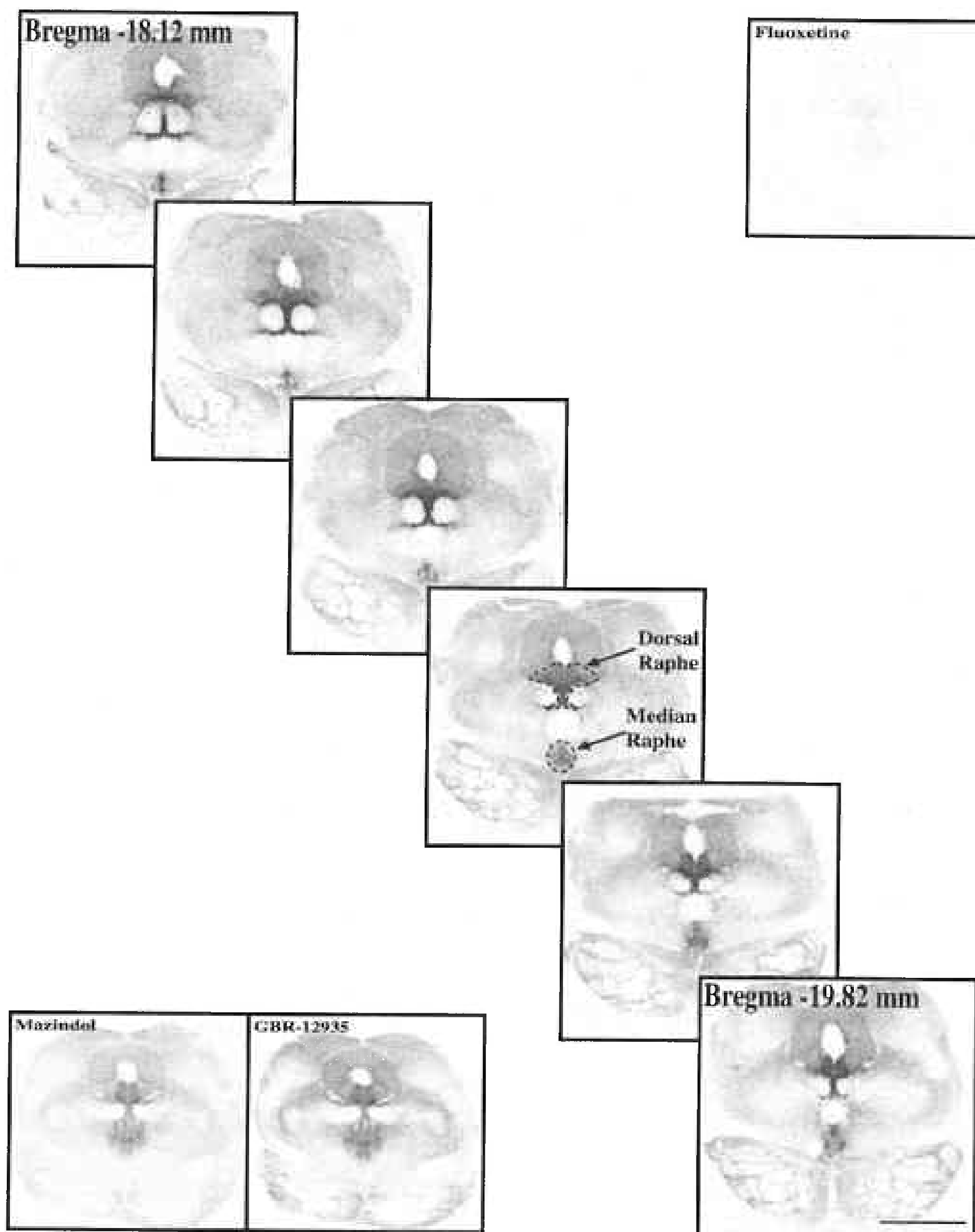


Figure 4-1. Autoradiograms of [^{3}H]citalopram binding to monkey midbrain sections (10 μm). Midbrain sections through six levels of the DRN and MRN, with an interval of 300 μm, are shown. [^{3}H]Citalopram binding was dense in the DRN, moderate in the median raphe nucleus (MRN) and light in the periaquiductal gray. Fluoxetine (0.1 μM) effectively blocked SERT labeling. Mazindol and GBR-12935 (0.1 μM) did not affect SERT labeling. Scale bar=5 mm.

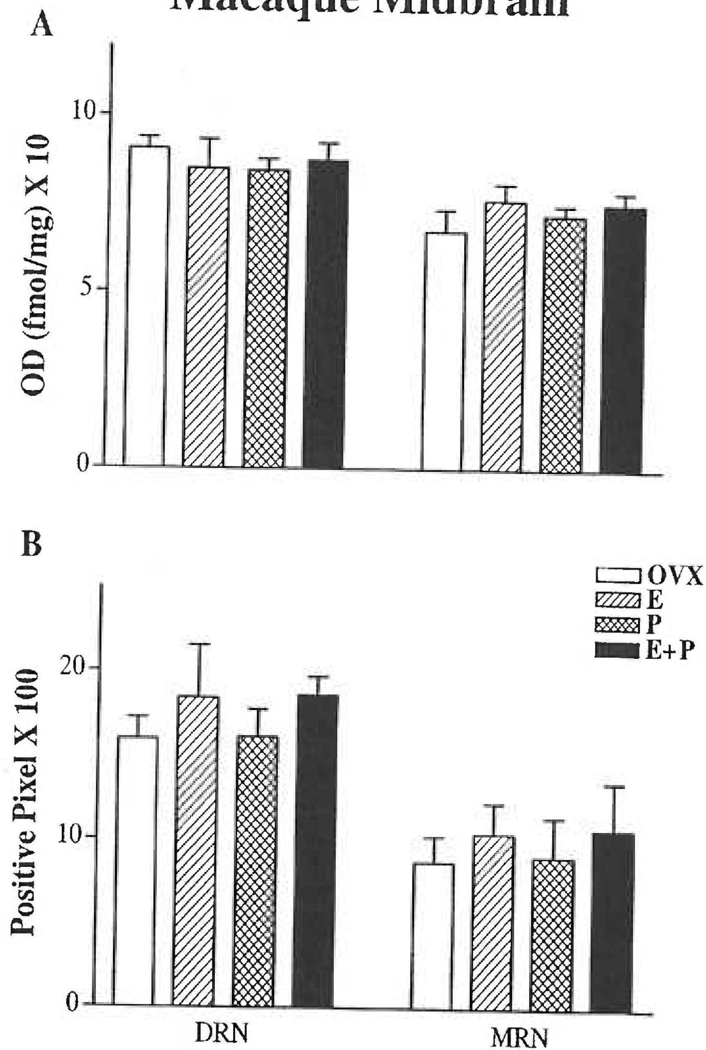
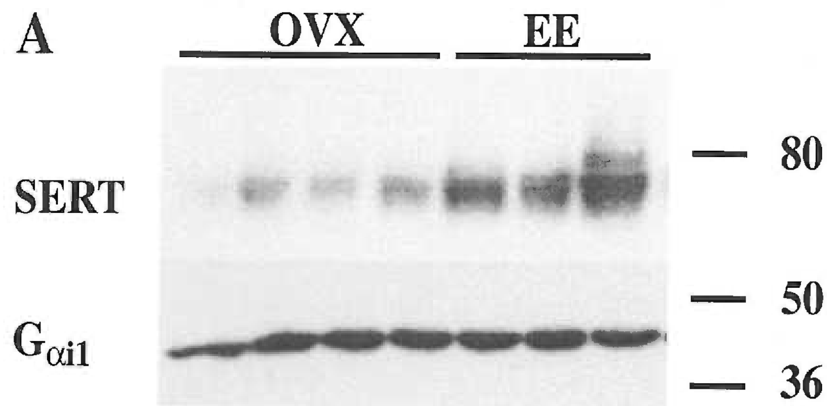


Figure 4-2. Comparison of [^{3}H]citalopram binding in the monkey raphe from OVX, E (28 d), P (28 d), and E (28 d) plus P (last 14 d) treated macaques. **A**. Average OD values from six levels of the DRN and MRN (n=5/treatment). Compared to the OVX control animals, SERT binding sites were not changed by E, P, and E+P (ANOVA, p>0.05). **B**. Positive pixel areas reflecting [^{3}H]citalopram binding in the DRN and MRN. Compared to the OVX control animals, [^{3}H]citalopram-generated pixel areas in the DRN and MRN were not affected by any of the treatments (ANOVA, p>0.05).



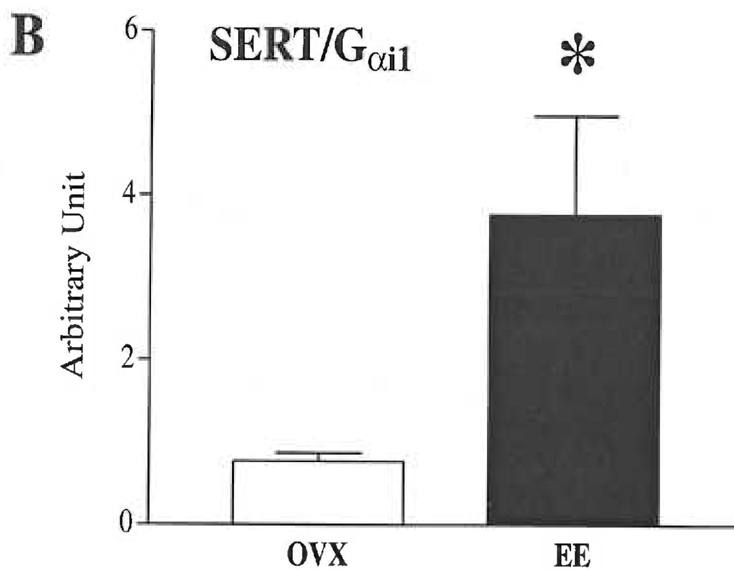


Figure 4-3. Equine estrogen (EE, 30 months) significantly increased SERT protein levels. **A**. SERT protein signal bands (78 kd) detected by Western blot analyses. **B**. Densitometric analysis showed a significant increase of the SERT band density, normalized with $G_{\alpha i1}$, by EE treatment compared to the control group with unpaired two tailed t test (p=0.0312, t=2.969, df=5). * Significantly different from the OVX control group.

Monkey SERT Degradation at 37C

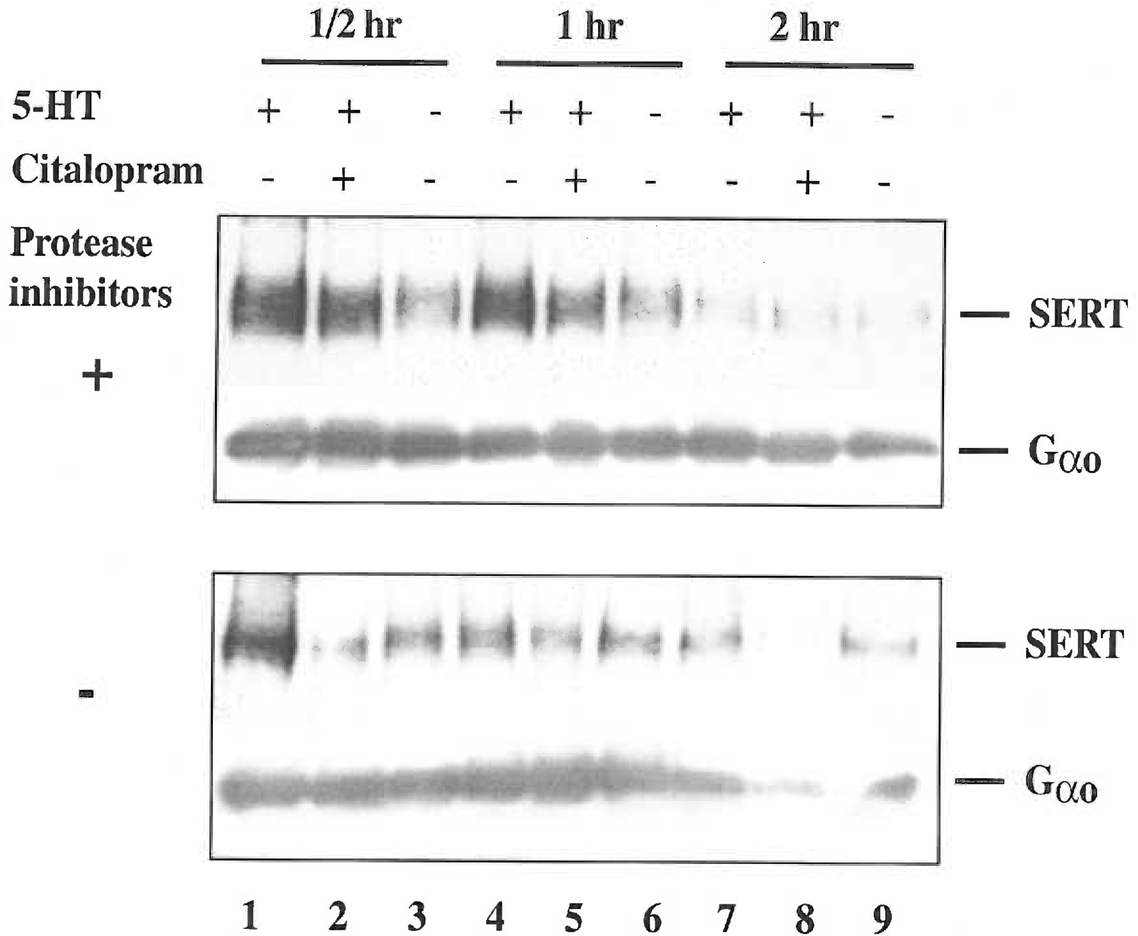


Figure 4-4. Western analysis showed that SERT degradation was inhibited by added serotonin (0.4 nmol/μg protein) *in vitro*. The same amount of monkey midbrain extracts were incubated with (lanes1, 2, 4, 5, 7, and 8) or without (lanes 3, 6, and 9) added serotonin for 30 to 120 min in the absence or presence of protease inhibitors before Western analysis. Citalopram (0.4 nmol/μg protein) blocked the effect of sertonin when protease inhibitors are abscent (lanse 2, 5 and 8).

[^{3}H]Citalopram Binding in the Macaque Hypothalamus

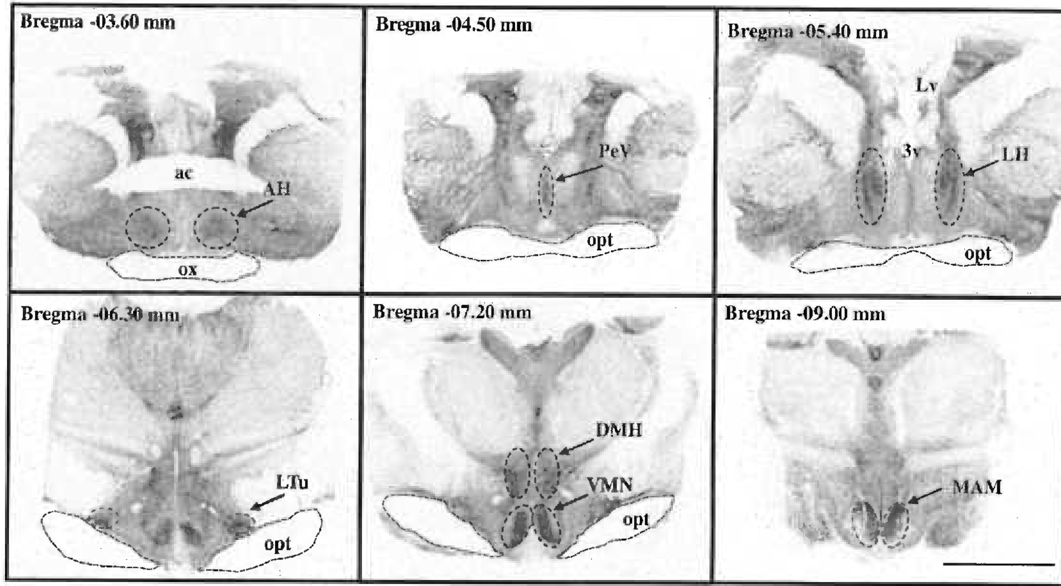


Figure 4-5. Autoradiograms of [^{3}H]citalopram binding to monkey hypothalamic sections (10 μm). The distribution of SERT binding sites in the hypothalamus is widespread, yet discrete labeling was found in the anterior hypothalamus (AH), periventricular nuclei (PeV), lateral hypothalamus (LH), lateral tuberal nuclei (LTu), ventromedial nuclei (VMN), dorsal medial hypothalamus (DMH), and MAM (mammilary body). 3v (third ventricle), ac (anterior commissure), opt (optic tract), ox (optic chiasm), and Lv (lateral ventricle). Scale bar=5 mm.

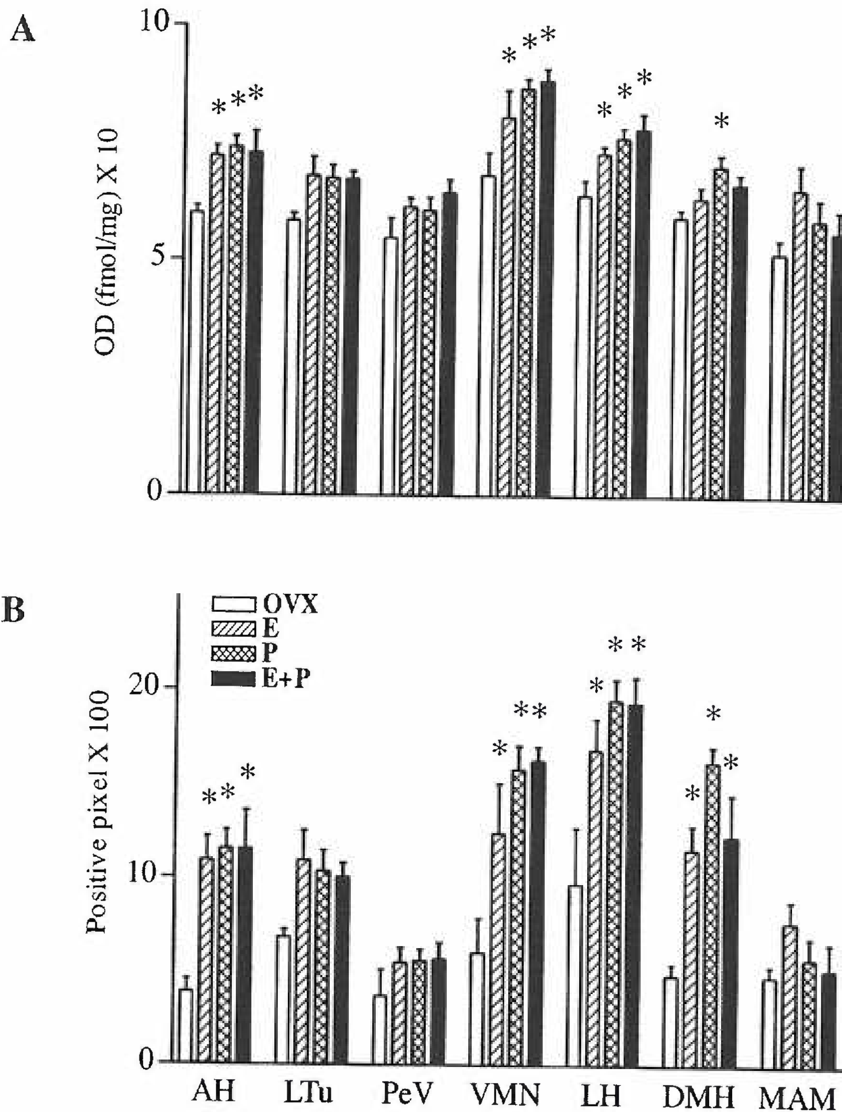


Figure 4-6. Comparison of [^{3}H]citalopram binding sites in monkey hypothalamic sections from OVX, E (28 d), P (28 d), and E (28 d) plus P (last 14 d) treated macaques. **A**. Average OD values from four to six levels of each hypothalamic nucleus (n=5/treatment). Compared to the OVX control animals, SERT binding sites were significantly increased by E, P, or E+P in the AH (ANOVA; p=0.028, F=4.008, df=18), VMN (p=0.014, F=4.931, df=18), LH (p=0.011, F=5.236, df=18), and by P in the DMH (p=0.027, F=4.035, df=18). **B**. Positive pixel areas reflecting [^{3}H]citalopram binding in the hypothalamus. Compared to the OVX control animals, [^{3}H]citalopram-labeled positive pixel areas were significantly increased by E, P, or E+P in the AH (ANOVA; p=0.006, F=6.183, df=18), VMN (p= 0.005, F=6.449, df=18), LH (p=0.006, F=6.129, df=18), and DMH (p=0.001, F=9.806, df=18). [^{3}H]Citalopram binding sites were not changed by any of the treatments in the LTU, PEV, or MAM. * Significantly different from the OVX control group.

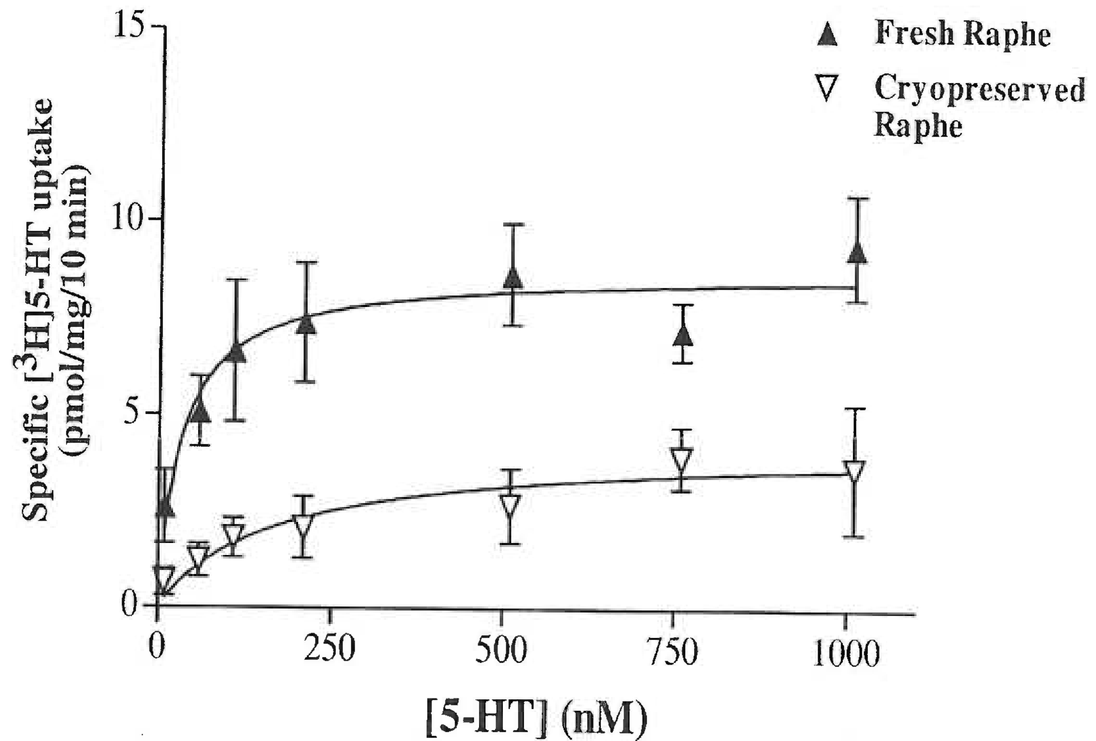


Figure 4-7. Specific serotonin uptake by synaptosomes prepared from fresh and cryopreserved monkey dorsal raphes. Km values for these two preparations were similar. Vmax value of cryopreserved tissue reached about half of the Vmax value obtained from fresh tissue. Subsequently, serotonin uptake by synaptosomes prepared from cryopreserved brain blocks of twenty animals from four different treatment groups was determined.

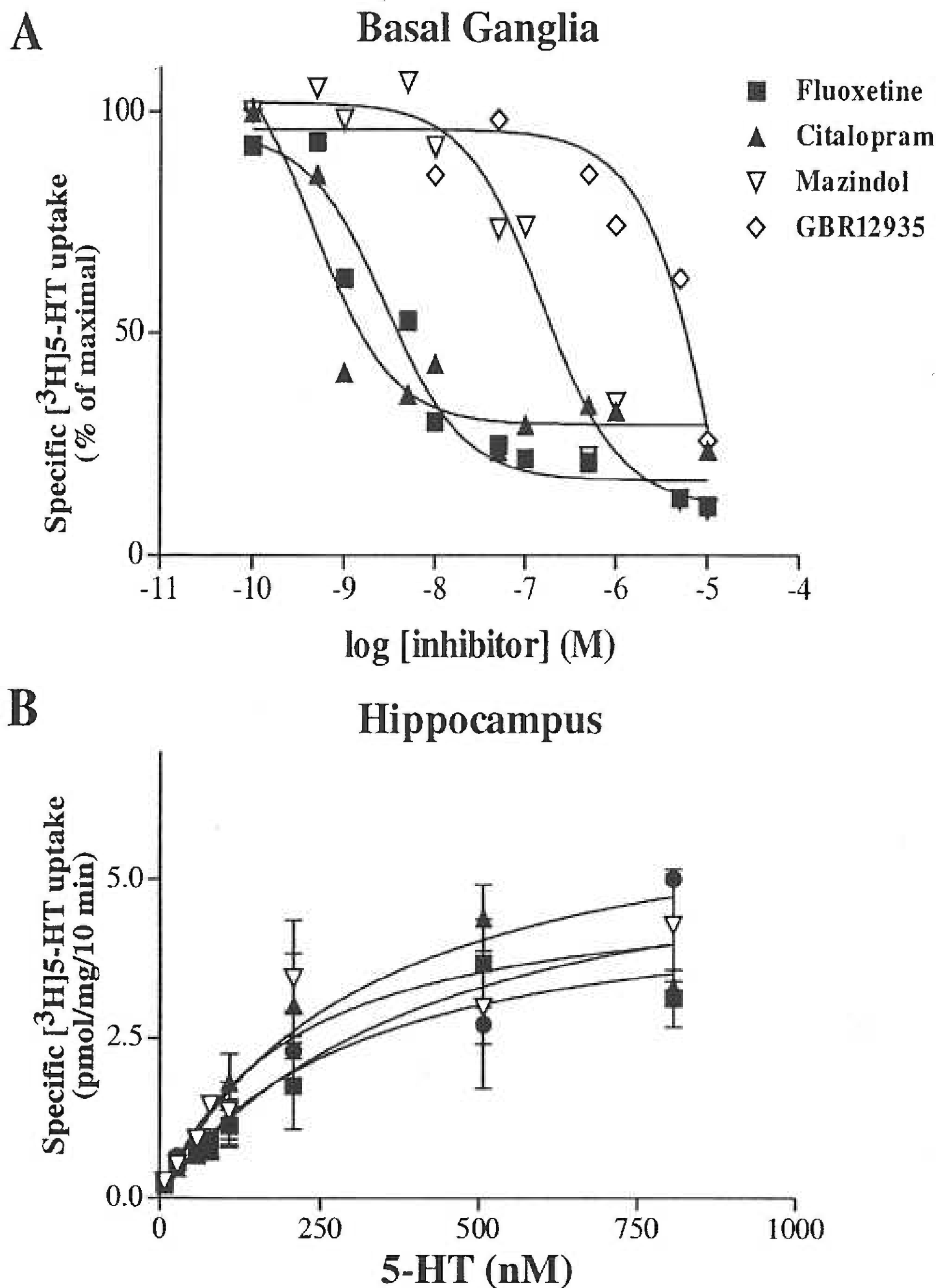


Figure4- 8. Demonstration of the specificity of reuptake. **A**. Serotonin uptake assay selectively measured the transport via SERT. The IC_{50} values for citalopram, fluoxetine, mazindol, and GBR-129-35 at blocking serotonin uptake in the basal ganglia corresponded with the rank orders of their affinities for SERT, i.e., citalopram > fluoxetine >> mazindol >> GBR-12935. **B**. Specific serotonin uptake was measured and compared in the basal ganglia (not shown) and hippocampi of OVX, E, P, and E+P treated macaques. A model for one-site best described the saturable transport of serotonin.

CHAPTER 5 Summary and Conclusions

In summary, one month of hormone replacement downregulated 5-HT_{1A} autoreceptor binding, which reflected the downregulation of the autoreceptor mRNA, and autoreceptor mediated G protein activation and cell signaling without affecting the intrinsic property of the receptor in non-human primates. Down-regulation of receptor protein levels and $G_{\alpha i3}$ may have co-contributed to the downregulation of the receptor function. These data indicate that ovarian hormones may stimulate serotonin neurotransmission, in part, by decreasing the level of 5-HT_{1A} receptors and inhibitory G proteins utilized for intracellular signaling. In addition, postsynaptic 5-HT_{1A} binding density in multiple hypothalamic nuclei is also downregulated by the same hormonal treatment, enabling a disinhibition in the host cells of these postsynaptic 5-HT_{1A} receptors.

However, hormone replacement in non-human primates did not change SERT binding sites in the raphe, but it increased SERT protein mass at the somatodendritic location and increased the density of SERT binding and serotonin uptake in selective serotonergic terminal fields without affecting the affinity of the transporter. These changes at the SERT protein and functional level did not reflect changes in SERT gene expression observed in previous studies. Rather, the up-regulation of SERT binding and uptake in selected brain areas may have resulted from the elevated extracellular serotonin during hormone replacement.

Estrogen and Progestin in the Brain

Compelling evidence has been gathered to demonstrate various beneficial effects of E in the brain during development, puberty, adult life, and senescence. However, several physiological endpoints indicate that P supplemented to E stimulates

brain serotonin tone further than E alone and studies on the mechanisms of actions of P in the brain are scarce (Bethea et al. 2002a). This study showed that at protein and functional levels of 5-HT_{1A} and SERT, E and P did not have an additive effect. Moreover, the actions of both E and P were gene and brain region specific, which may be due to the cell context of ER and PR, the transcription factors mediating the actions of E and P, respectively. For example, monkey serotonin neurons express ERβ and PR whereas the predominant combination in serotonin target neurons in the hypothalamus is ERα and PR. Therefore, P alone modulated both 5-HT_{1A} autoreceptor binding in the raphe and SERT binding in the hypothalamus, both of which are mediated by PR in the company of ERβ in serotonin cells. However, P alone did not regulate postsynaptic 5-HT_{1A} binding in the ERα and PR containing non-serotonin neurons in the hypothalamus. ER and PR share and compete for co-regulatory proteins in the same cell (Shibata et al. 1997). Through complex protein-protein interactions, ERα and ERβ may affect the action of PR differentially in serotonin and non-serotonin neurons.

Ovarian hormones as Antidepressants

Currently used antidepressants include SSRIs such as fluoxetine (PROZAC), monoamine inhibitors such as phenelzine (NARDIL), and tricyclics such as amitriptyline (ELAVIL). The onset of the efficacy of all these compounds has a two to four-weeks delay after administration. Therefore, poor compliance, along with high cost and untoward cardiovascular, sleep and sexual side effects, have limited the clinical usage of these drugs. Thus, new treatment regimens for depression have been constantly sought.

Findings from this project indicated that ovarian hormones, E and P, stimulate serotonin neural transmission and thereby, may have beneficial effects in treating

depression and other serotonin-related psychiatric disorders. A recent preliminary clinical study of depressed perimenopausal women reported an 80% response rate to E treatment compared to a 22% response rate to placebo administration (Schmidt et al. 2000). The average response rate to SSRIs and other antidepressants is 70% (Moller, 2000), comparable to that of E.

Previous reports from this laboratory indicate that addition of P does not alter the stimulatory effect of E in serotonin neurons. In reproductive tissues, for example the uterus, the physiological function of P is antagonistic to that of E (Brenner, Maslar, 1988). This action of P is the basis of supplementing E replacement therapy in postmenopausal women with P to prevent endometrium hypertrophy. However in the brain, P is synergistic to E's action as evidenced by the neuroendocrine regulation of gonadotropin surge and prolactin secretion (Bethea et al. 2002a). One of the explanations for the tissue specific effect of P is the tissue-specific composition of PR isoforms. In the CNS (e.g. the hypothalamus) of macaques, the ratio of PR-A/PR-B is close to 1 whereas in the uterus, this ratio is above 5 (Bethea, Widmann, 1998). PR-A is the dominant negative inhibitor between the two PR isoforms. The findings of this study and others further support the notion that P is not always antagonistic to E in the brain and compounds targeting at PR could be new candidates for antidepressant development.

Various synthetic forms of E and P are being prescribed to women in the clinic, but little is known of their actions in the brain. The most-commonly used synthetic progestin in the clinic, medroxyprogesterone acetate (MPA), does not protect against coronary artery hyperreactivity as natural P does (Minshall et al. 1998). In addition, MPA blocked the E-induced protein expression of TPH in the macaque whereas natural P did not block the stimulatory effect of E on the expression of the same enzyme (Bethea et al. 2000). In terms of E, selective estrogen receptor modulators

(SERMs) are needed to preserve the beneficial effect of E in the bone, heart, and brain while obliterating the hyperplasic effect of natural E in reproductive tissues.

Hormone Replacement as an Adjuvant Therapy to SSRI

It is has been suggested that hormone replacement could become a beneficial adjuvant therapy to current prescribed SSRIs (Stahl, 2001). The regulatory effects of ovarian hormones in serotonin system may stimulate serotonin neurotransmission in aspects not regulated by SSRIs. In the DRN of depressed patients, SERT mRNA expression is high yet SERT protein availability is low which is accompanied by low serotonin levels in these patients (Arango et al. 2001). The majority of studies investigating SSRI regulation of SERT expression reported no modification either at the message level or at the protein level (Lesch et al. 1993; Spurlock et al. 1994; Neumaier et al. 1996; Benmansour et al. 1999; Graham et al. 1987; Cheetham et al. 1993; Gobbi et al. 1997). As evidenced here and in previous studies, hormone replacement reduces SERT mRNA expression while preserving SERT protein expression, possibly due to high synaptic serotonin level. In addition, two other serotonin neuron specific genes, 5-HT_{1A} and TPH, are regulated by E and P but not by SSRIs (Bethea et al. 2002a). Together, these data predict a possible high response rate and fast onset when hormone replacement is supplemented to SSRI regimen. Furthermore, The addition of hormones to the SSRIs may also ameliorate the sexual side effects of SSRI compounds. A large-scale clinical study comparing the efficacy of SSRI with or without ovarian hormone supplementation in depressed women is warranted (Bethea et al. 2002a).

Side effects of hormone replacement include breakthrough bleeding, nausea, and development of uterine and breast cancer in high risk group patients. In order to preserve the beneficial effects of the ovarian hormones and lower the side effects of

hormone replacement therapy, several new SERM compounds such as raloxifene have been developed. The Study of Tamoxifen and Raloxifene (STAR), a five year project that began in July 1999 at almost 500 centers in North America with 22,000 postmenopausal women participants, is aimed to reveal the benefits and pitfalls of these SERM compounds. In monkeys, raloxifene and arzoxifene (a newer SERM), act in a manner similar to natural E on TPH and SERT gene expression in serotonin neurons (Bethea et al. 2002b). Raloxifene, but not tamoxifen, acted like natural E on 5-HT_{2A} receptor density and expression in rat forebrain (Cyr et al. 2000). These studies promise an efficient hormone replacement regimen for SSRI supplementation in the near future.

The Regulation of Serotonin Reuptake Transporter

SERT expression is regulated at multiple cellular levels. At the gene expression level, several cell signaling pathways converge at specific DNA sequence in the promoter regions of the SERT gene (Blakely et al. 1996). These modulatory sequences include CRE, AP1, AP2, α-INF, γ-IRE, NF-IL6, NF-κB, and SP1 sites. Therefore, serotonin neurons are responsive to multiple environmental stimuli that trigger the various cell signaling networks. For example, serotonin neurons receive neronal inputs from glutamatergic, GABAergic, and adrenergic neurons as well as the autocrine feedback of serotonin. The precise interplay among different signaling pathways in regulating SERT gene expression is not understood. Less defined is the role of ovarian hormones in regulating these different signaling systems.

Findings of this study also indicated that the trans-Golgi network where functional SERT proteins are modified, sorted, and trafficked to the selected cell membrane locale may be another important cellular component regulated by ovarian hormones. SERT expression and regulation during hormone replacement is brain

region specific. However, our knowledge in SERT sorting and trafficking is mostly speculative. Cell surface expression of SERT proteins displays a polarized pattern, i.e. concentrated at cell axon and dendrites but not at nerve terminals of neurons and basolateral domains of polarized epithelial cells MDCK and LLC-PK1 (Gu et al. 1996). At specialized zones in the plasma membrane, SERT proteins are expressed through docking of the carrier vesicles prior to fusion through noncovalent linkages between vesicular proteins (v-SNARES) and plasma membrane targets (t-SNAREs) (Bajjalieh, Scheller, 1995; Pfeffer, 1996). The t-SNARE protein SNAP-25 has a similar polarized distribution to that of SERT to in adrenal chromaffin cells (Blakely et al. 1998). Ovarian steroid regulation of SERT sorting and trafficking is a brand new, yet exciting research area.

In general, protein expression is conventionally thought to reflect the level of gene expression. However, accumulating data are demonstrating that SERT expression during hormone replacement may be an exception. As mentioned earlier, SERT protein availability reflects the synaptic serotonin level reliably in depressed patients (Malison et al. 1998; Gross-Isseroff et al. 1998; Rausch et al. 1982; Faludi et al. 1988; Mann et al. 1992; Halbreich, Tworek, 1993; Cleare, 1997), in individuals with homozygotic "Short" polymorphic forms at SERT alleles (Lesch et al. 1996; Stober et al. 1996), and in ovariectomized monkeys compared to those treated with hormones (Chapter 4). In addition, this positive correlation between the transporter protein expression and synaptic neurotransmitter levels is being recognized as a general phenomenon in the Na+/Cl- dependent neurotransmitter transporter family including the SERT, norepinephrine transporter and others (Apparsundaram et al. 2001).

How can serotonin neurotransmission be stimulated during hormone replacement when SERT proteins are abundant? Recent kinetic studies reported that the rate of neurotransmitter transport by a SERT protein approximates 1 serotonin

molecule per second (Mager et al. 1994), hundred to thousand fold slower than the rate of serotonin triggering a receptor response initiation by serotonin. For example, 5-HT_{1A} mediated inwardly rectifying K^+ current can be detected within 30 ms of receptor stimulation (Karschin et al. 1991). The response rate for the serotonin gated ion channels (5-HT_3 receptors) is within ms range (Barnes, Sharp, 1999). The dynamic picture in a serotonin synapse during hormone replacement therefore, involves a high serotonin level, a backed up clearance through the transporter, and a fast and long-lasting cellular signaling through various serotonin receptors. Thus, the findings of this study and others support the notion that SERT might not merely be a housekeeper at serotonin synapses to recycle serotonin but also a substrate dependent sensor to feedback to serotonin cellular activity, such as SERT gene transcription. This implication is in agreement with the observation that excitatory amino acid transporters function more as a substrate dependent Cl^- channel to regulate membrane conductance than as a conventional neurotransmitter transporter (Fairman, Amara, 1999). Consistent with this hypothesis is that SERT is localized at the perisynaptic region instead of being within the terminals (Blakely et al. 1998).

Ovarian Hormones and Cell Signaling

The observation that $G_{\alpha i3}$ in the monkey midbrain DRN is downregulated during hormone replacement provides a lead to further investigation of steroid regulation of components of G protein signaling system. Regulators of G-protein-signaling (RGS proteins) are particularly interesting because of their important roles in G protein activities. At least 24 mammalian RGS proteins have been cloned and the number of this family of proteins is ever increasing (Ross, Wilkie, 2000). All members of RGS proteins regulate G protein signaling via their GTPase activating activity. The intimate relationship between RGS proteins and subunits of G proteins as well as the

great variety among members of this superfamily of regualtory protein makes it attractive for steroid regulation studies. In addition, protein kinases such as PKA and PKC all have been shown to cross-talk with steroid hormones (Kelly et al. 1999). Thus, the regulation of multiple cellular pathways by E and P at multiple levels is likely to coexist in serotonin neurons.

It is possible that the actions of steroids at different pathways occur in a temporally coordinated fashion. For example, E-stimulated PKC activity and 5-HT_{1A} desensitization occurs within minutes of drug administration and lasts for up to two or three hours (Mize et al. 2001; Raymond et al. 1999). However in this study, the downregulation of 5-HT_{1A} by E and P is observed after a month of hormone replacement. Therefore, coordinated recruitment of various cellular pathways might be a general theme during E and P stimulation of serotonin neurotransmission.

Summary

Together with other observations, this research demonstrated that ovarian hormones target and regulate the central serotonergic activity. Through the synergy of multiple steroid sensitive pathways, serotonin neurotransmission is very likely to be stimulated during hormone replacement. Figure 5-1 provides a schematic summary of our current knowledge of components of serotonin neurotransmission regulated by ovarian hormones.

1. TPH is upregulated at the gene and protein level, indicating a greater serotonin production in serotonin cells.

2. Downregulation of 5-HT1A gene and protein expression and $G_{\alpha i3}$ levels contribute to lowered activity of 5-HT_{1A} autoreceptors, enabling a disinhibition of serotonin cell firing and serotonin release.

3. MAO-A gene expression is downregulated in serotonin cells and serotonin-target neurons, indicating a slower metabolism of serotonin during hormone replacement.

4. Serotonin transport through SERT proteins at serotonin nerve terminals is accelerated due to the increase of the number of functional SERT proteins. The increased SERT activity may feedback and downregulate SERT gene expression.

5. Postsynptically, serotonin target cells respond to E and P treatment in a region specific manner. For example, both 5-HT_{1A} postsynaptic receptor density and 5-HT_{2C} gene expression are downregulated in the VMN but not in POA of the hypothalamus. Different compositions of nuclear steroid receptors in different target neurons may account for the region specificity.

6. E + P act in serotonin neurons to both increase and decrease gene expression, indicating the presence of different mechanisms of gene transcription regulation by steriods. NF-κB, a transcription factor that can be sequestered by ER and PR, translocates to the cytoplasm during hormone replacement and may reduce the activation of genes regulated by NF-κB (Bethea et al. 2002a).

7 & 8. Ovarian hormones may affect protein sorting and trafficking in serotonin neurons and efferent inputs from other neural systems.

Future Directions

The data in this thesis have furthered our understanding of the role of ovarian steroids in serotonin neural system. In addition, several future directions can be derived from this study. For examples, Clinical experiments are warrated in order to investigate the onset rate and efficiency of fluoxetine in the presence and absence of natural E and P in depressed postmenopausal women. SSRI antidepressants need to

be taken two to four weeks to manifest their efficacy. Our current knowledge predicts a shorter onset rate of the SSRIs when supplemented with female hormones.

In addition, cDNA array analysis of gene expressions in serotonin neurons of placebo, E, and E + P treated OVX macaques may be performed. Serotonin neurons can be harvested from midbrain sections using Laser Capturing Microscopy after immunocytochemical labeling. Such experiments will reveal, in serotonin neurons, components of the G protein system, protein kinases involved in cell signaling, and gene transcription factors that are affected by ovarian hormones.

Furthermore, experiments to survey the promoter region of SERT and 5-HT_{1A} genes are warranted using site-directed mutagenesis and deletion to locate the sequences responsible for the E mediated inhibition of these two genes. HEK-293 would be an optimal cell line for these experiments. They are of human embryonic kidney origin and devoid of endogenous expression of SERT, 5-HT_{1A} and steroid receptors and HEK-293 cells stably transfected with SERT are available. Estrogen response elements have not been detected in either SERT or 5-HT_{1A} promoters. However, several consensus regulatory sites such as NF-κB response elements and AP-1 sites are present in the promoter region of these two genes. Results from these experiments could provide the basis for new mechanisms of gene inhibition by steroid hormones.

Experiments may also be performed to test the hypothesis that ERβ sequesters NF-κB to inhibit 5-HT_{1A} gene expression. RN46A cells are an immortalized serotonin cell line in which both 5-HT_{1A} and ERβ, but not ERα, are expressed and they would be an optimal testing system. ERβ has been shown to sequester NF-κB and inhibit tumor necrosis factor-alpha transcription more efficiently than ERα in U937 cells (An et al. 1999). The regulation of 5-HT_{1A} transcription will be compared when NF-κB signaling is stimulated in the presence and absence of E.

It is also of interest to test the hypothesis that 5-HT$_{1A}$-mediated decrease of cAMP production in the presence of E represses SERT transcription. Transcription factor CREB proteins, when phosphorylated by PKA and bound with the cAMP response element in the SERT promoter region, increases SERT transcription (Blakely et al. 1996). During hormone replacement, elevated serotonin stimulates 5-HT$_{1A}$ to decrease intracellular cAMP levels and PKA activity, which in turn may decrease SERT transcription. In RN46A cells, cAMP assay will be developed and the cAMP levels will be compared before and after E treatment.

In conclusion, the serotonin neural system regulates mood, integrative cognition, sexual behavior, satiety, hormone secretion and numerous other autonomic neural functions. This thesis has shown that the ovarian hormones, E and P, regulate two critical components of serotonin neurotransmission, the 5-HT$_{1A}$ autoreceptor and SERT. Thus, a mechanism by which female hormones can impact autonomic, limbic, and higher neural function has been elucidated. Moreover, future directions for research into the cellular signal transduction mechanisms influenced by E and P are suggested and immediate clinical studies in humans are indicated.

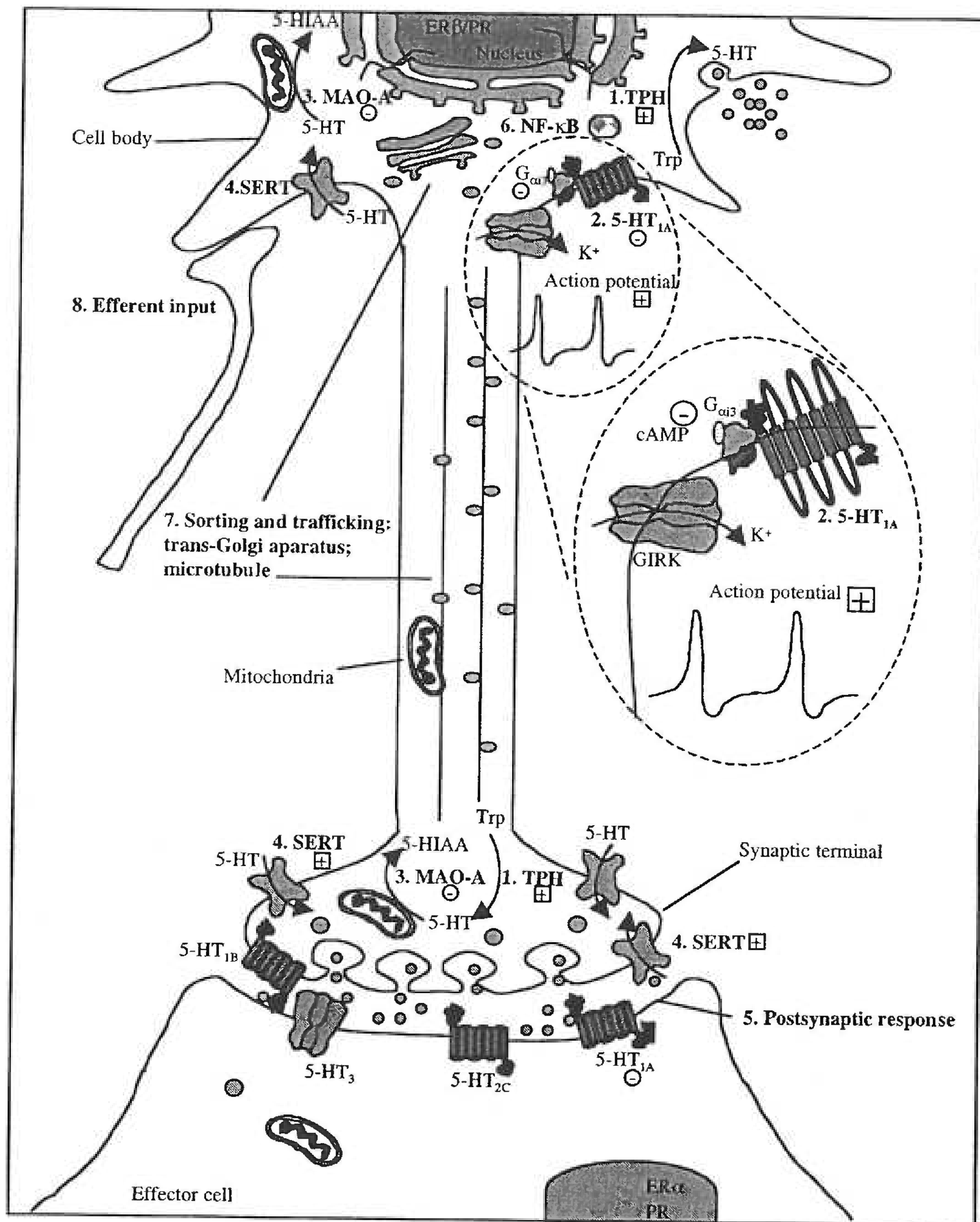


Figure 5-1 E and P regulate multiple components of serotonin neurotransmission. 1. TPH, tryptophan hydroxylase; 2. 5-HT_{1A} autoreceptors; 3. MAO-A, monoamine oxidase-A; 4. SERT, serotonin reuptake transporter; 5. Postsynaptical target cells; 6. Transcription factors such as NF-κB; 7. Sorting and trafficking; 8. Efferent inputs from other neural systems. 5-HIAA, 5-hydroxyindolacetic acid; 5-HT, serotonin; ER, estrogen receptor; GIRK, G protein coupled inwardly rectifying K^+ Channels; PR, progestin receptor.

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