CHAPTER II
LITERATURE REVIEWS

A. Anxiety

1. Fear

An animal's ability to experience fear is essential for its survival. Fear triggers the familiar "fight or flight" response, characterized by increased heart rate, breathing, and muscle tension, which allows the individual to escape from danger or defend itself against a predator. Experiments involving animals have indicated that information about threatening stimuli is transmitted to the lateral nuclei, the major input nuclei of the amygdala, which in turn project both directly and indirectly to the central nucleus, the major output nucleus of the amygdala. Efferent projections from the central nucleus activate numerous effector sites for physiological and behavioral fear responses (Figure 1).

Direct projections from the central nucleus of the amygdala to the lateral hypothalamus mediate the activation of the sympathetic autonomic nervous system that is seen during fear and anxiety. Projections to the dorsal motor nucleus of the vagus or parabrachial nucleus (Hopkins and Holstege, 1978) are involved in several autonomic measures of fear or anxiety. Projections to the ventral tegmental area (Beckstead *et al.*, 1979) may mediate stress-induced increases in dopamine (DA) metabolites in the prefrontal cortex. Projections to the locus coeruleus (Wallace *et al.*, 1989) may mediate activation of the locus coeruleus, which has been linked to fear and anxiety. Direct projections to the lateral dorsal tegmental nucleus and the parabrachial nuclei, which have cholinergic neurons projecting to the thalamus, may mediate increases in synaptic transmission in thalamic sensory relay neurons during states of fear. This, along with increases in thalamic transmission accompanying activation of the locus coeruleus, may lead to increased vigilance and superior signal detection in a state of fear. Release of norepinephrine (NE) or serotonin onto motor neurons, via amygdaloid activation of the locus coeruleus or raphe neurons, could lead to enhanced motor performance. The central nucleus of the amygdala projects to a region of the central grey implicated in conditioned fear in several behavioral tests. Direct projections to the trigeminal and facial motor nuclei may mediate some of the facial expressions of fear. Finally, direct projections to the paraventricular nucleus of

the hypothalamus, or indirect projections by way of the bed nucleus of the stria terminalis and preoptic area, may mediate the prominent neuroendocrine responses to fearful or stressful stimuli.

Under normal circumstances, these various physiological and behavioral responses are adaptive and enhance survival. Indeed, fear helps ensure that we avoid and escape from dangerous environments. However, it has been hypothesized that symptoms of anxiety disorders occur in response to the abnormal function of such fear circuitry.

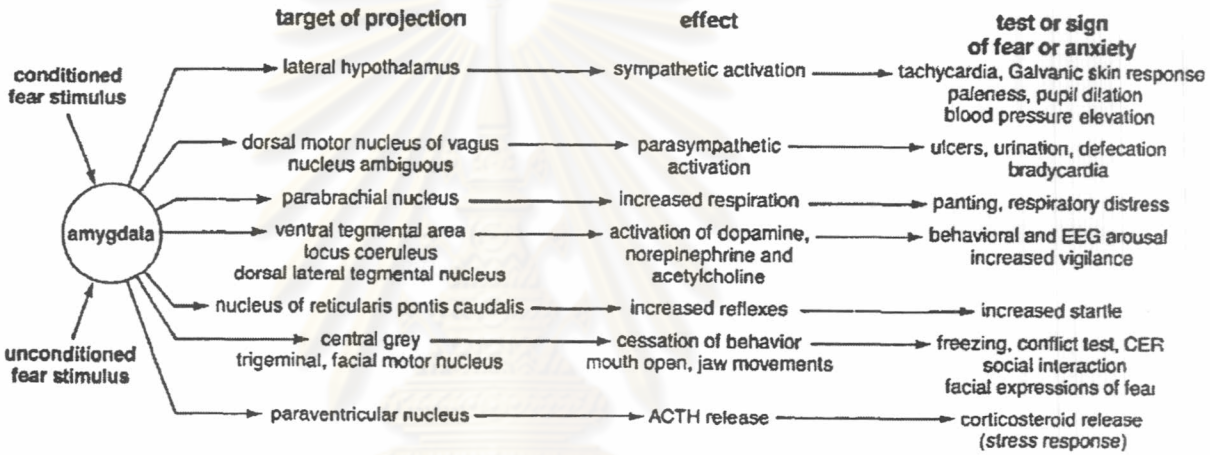

Figure 1. Diagram of direct connections between the central nucleus of the amygdala and a variety of hypothalamic and brainstem target areas that may be involved in different animal tests of fear and anxiety (Davis, 1992).

2. Anxiety disorders

The anticipation of danger, a characteristic of anxiety results in arousal, vigilance, physiological preparedness, and negative subjective states that are qualitatively similar to those associated with fear. Yet anxiety differs from fear in that it is triggered in the absence of an immediately threatening stimulus. Moreover, anxiety is often elicited by generalized cues, whereas fear is elicited by discrete, explicit cues. There are differences in anxiety disorders not just in time and intensity, but also in quality. These phenomenological differences to some extent are backed up by differences in physiological changes (Garvey *et al.*, 1987; Klein, 1993), biological markers, and pharmacological response and have led psychiatrists to a more detailed way to describe the individual symptoms of anxiety. This has led to a classification of

anxiety disorders based on symptom patterns. Taking into account the phenomenology (intensity, length, quality, and natural history of anxiety symptoms), along with differences in biology (genetics, physiological markers, and pharmacological responses), psychiatrists have constructed syndromes that now constitute a widely accepted typology. The fourth edition of the Diagnostic and Statistical Manual (DSM-IV) of the American Psychiatric Association describes several syndromes, consists of PD, agoraphobia, social phobia, obsessive-compulsive disorder, posttraumatic stress disorder, acute stress disorder, and GAD.

Panic disorder presenting panic attacks with intense anxiety accompanied by somatic symptoms such as tachycardia, tachypnea, and dizziness, is diagnosed when multiple panic attacks occur or when one or a few attacks are followed by persistent fear of having another attack.

Agoraphobia has been described as fear of open spaces. In its severest form, agoraphobia is characterized by a paralyzing terror of being in places or situations from which the patient feels there is neither escape nor accessible help in case of an attack. Consequently, people with agoraphobia confine themselves to places in which they feel safe, usually at home.

Social phobia is a persistent fear of one or more social situations involving possible exposure to scrutiny by others, and associated fear of humiliation. An example of a specific social phobia is stage fright. A social phobia may generalize, in which case it may lead to avoidance of all social situations, resulting in substantial social and occupational disability. The boundary between shyness and this type of social phobia has not been well clarified.

Obsessive-compulsive disorder (OCD) involves either 1) recurrent intrusive thoughts that an individual recognizes as products of his or her own mine (obsessions); or 2) repetitive, seemingly purposeful behavior designed to prevent or neutralize a dreaded occurrence, often as a consequence of the obsession (compulsions); or both.

Posttraumatic stress disorder (PTSD) has occurred often after serious trauma and is characterized by numbing, cue-elicited reliving of the traumatic experience, increased startle, and nightmares.

Acute stress disorder has been identified as a syndrome in which symptoms of PTSD occur within two days to four weeks after the traumatic event.

Acute stress disorder can accurately identify up to 94% of victims at risk for PTSD, and between 50% and 80% actually develop the more chronic and serious disorder.

Generalized anxiety disorder is characterized by unrealistic and excessive worry for more than six months accompanied by specific anxiety-related symptoms, such as motor tension, sympathetic hyperactivity, and excessive vigilance.

2.1 Neuroanatomy of anxiety

Evidence about the neuroanatomy of anxiety comes from animal experiments (including stimulation and ablation works) and human studies using static (magnetic resonance imaging) and functional (positron emission tomography/single photon emission computerized tomography) neuroimaging.

Two important concepts underlie the functional anatomy of anxiety

(1) The various types of anxiety can be broadly divided into different systems; a defense system and a behavioral inhibition system.

The defense system (reviewed by Misslin, 2003) is directed towards making immediate responses to internal and external threats, loosely analogous to flight and fight (and, hence, to panic attacks). The behavioral inhibition system (Gray, 1988) is involved in suppressing behaviors that might increase danger, including at times the defense system. This is analogous to anticipatory anxiety, or to freezing in animal models.

(2) The neural circuits that underlie anxiety are organized at different levels, reflecting the different demands for cognitive processing.

For example, simple automatic responses are mediated by lower areas (i.e. PAG and locus coeruleus); above these are the intermediate levels (i.e. amygdala and septo-hippocampal systems) that mediate more practiced responses (reviewed by Pratt, 1992). Higher cortical regions (i.e. paralimbic cortex) manage more cognitively demanding stimuli (reviewed by Pratt, 1992). Each system has the ability to feedback onto those below it.

The brainstem areas are responsible for the physiological correlates of anxiety, fear, and panic. The PAG receives descending afferents from the paralimbic cortex and limbic systems, as well as ascending afferents from deep sensory structures. Stimulation of the ventrolateral PAG elicits freezing, hypotension, bradycardia, and analgesia, an adaptation to deep injury. Stimulation of the lateral

PAG produces flight caudally and fight rostrally, both accompanied with tachycardia and increased blood flow. This might explain, at least in part, the variety of symptoms that can be experienced as part of anxiety and panic. From results with electrical stimulation and intracerebral drug injection into the PAG indicated that serotonin inhibited aversive behavior evoked from this area (Schütz *et al.*, 1985; Vargas and Schenberg, 2001).

The locus coeruleus, a noradrenergic nucleus located lateral to the PAG, has widespread efferents to the cortex, limbic areas, and thalamus (Valentino and Aston-Jones, 1996). Electrophysiological and pharmacological stimulation causes an anxiety response (Redmond, 1985). This area has been heavily implicated in the genesis of panic from both pharmacological and anatomical work. Direct stimulation of the locus coeruleus in animals leads to behavior similar to human anxiety (Redmond and Huang, 1979; Redmond, 1985), which can be blocked by locus coeruleus lesions and anti-adrenergic and anxiolytic drugs (Uhde *et al.*, 1984).

The hypothalamus receives inputs from the limbic system and the locus coeruleus, and as such, has a key role in coordinating the neuroendocrine response to anxiety; namely, the activation of the hypothalamic-pituitary-axis (HPA)/sympatho-adrenal system, through the release of peptides and releasing hormones. This is an important common pathway in all stress (Selye, 1956) and anxiety reactions, including chronic stress and depression (LeDoux, 1998).

Clearly, the limbic system has a central role in the genesis of emotions, especially anxiety. Within the limbic system lies the amygdala, it has become a focus of interest (Davis, 1992). The amygdala has extensive communications, as well as interconnections to the cortex and locus coeruleus and projections to the striatum, midbrain, and brainstem. This means that it exerts control over locomotor, neuroendocrine, autonomic, and respiratory responses. Lesion, stimulation, and neurochemical studies consistently have implicated the amygdala in the expression, conditioning, and extinction of fear or acute anxiety (LeDoux, 1992). Increasingly, the amygdala is being seen as the common pathway and processor of fear. Pathways from the thalamus and cerebral cortex to the lateral nucleus of the amygdala are processed within the complex intra-amygdala circuitry. Efferents then go to the PAG, brainstem, and hypothalamus, through which the physiological response is mediated. The amygdala not only detects and organizes the responses to natural dangers, but

may also be the centre for learning about novel threats, so-called classical conditioning (Adamec, 1993; LaBar and LeDoux, 1996; LeDoux, 1996), this may be similar to kindling. The amygdala has an established sensitivity to kindling in the epilepsy, and it is interesting to note that a cross sensitization of defense behaviors may be induced by amygdala stimulation with a γ-aminobutyric acid (GABA) receptor agonist (Cahill and McGough, 1998). The amygdala, therefore, represents an important potential target for pharmacological agents (Gray, 1982). Excitatory amino acids mediate synaptic transmission in the amygdala from both cortical inputs and intrinsic amygdaloid connections. In this area, NMDA receptors seem to be involved in the formation of conditioned fear, whereas AMPA/kainate receptors appear to be involved in the expression of conditioned fear (reviewed by Walker and Davis, 2002).

Anxiety is more than a simple, stereotyped response to aversive stimuli, whether primary or conditioned. Animal and especially human learn about complex associations of adverse stimuli and are able to make discriminating responses. This requires a comparator system that can detect whether a threat is familiar, requiring a conditioned automatic response, or novel, and, hence, requiring higher order processing. The hippocampus has been consistently implicated in this role (Gabriel, 1993), with other accounts implicating the septum, posterior cingulate, and thalamic nuclei (Handley, 1995). Furthermore, it is also likely that different areas of the septo-hippocampal region may mediate different aspects of behavioral inhibition. The medial septal and dorsal hippocampus, for example, are involved in avoidance, as lesions to these areas release approach in conflict tasks (Fontaine *et al.*, 1990). These experimental findings are backed up by magnetic resonance imaging evidence of focal temporal lobe abnormalities, especially the right parahippocampal area, in patients with PD (McEwen and Sapolsky, 1995). In animals, sustained stress is known to cause degeneration in the hippocampus through chronically raised levels of glucocorticoids (Kim and Fanselow, 1992; Sapolsky, 1996), and impaired function of this area has a knock-on effect on conditioning to contextual cues (Bremner *et al.*, 1995; Philips and LeDoux, 1992). Work with Vietnam veterans suffering from PTSD has shown an 8% reduction in hippocampal volume. This raises the interesting idea that high levels of anxiety may be causative of structural brain damage, as well as secondary to it (Bridges *et al.*, 1973). In order to process more-demanding situations,

there must be some link between the sensory association, premotor and executive areas, and the limbic system. The paralimbic cortex (orbitofrontal, insular, anterior temporal, and anterior cingulate) provides this. Patients with lesions in these areas show decreased anxiety, and such lesions to the orbitofrontal and cingulate can be used as psychosurgical treatments for those with severe refractory anxiety (Ballantine *et al.*, 1987; Eison, 1989; Mindus and Jenike, 1992).

2.2 Pharmacology of anxiety disorders

The conclusion of the efficacy of various classes of anxiolytic drugs in different syndromes of anxiety disorders was shown in Table 1 (reviewed by Argyropoulos *et al.*, 2000).

Benzodiazepines (BDZs) have been the mainstay of pharmacological treatment of anxiety over the last 4 decades. BDZ is effective in the treatment of PD (Andersch *et al.*, 1991; Ballenger *et al.*, 1988) and GAD (Kahn *et al.*, 1986; Rickels *et al.*, 1993). However, the problems associated with their long-term use prompted the research for alternative agents that would be useful in anxiety conditions.

Antidepressants

- Tricyclic antidepressants (TCAs) have antipanic activity and indeed this response has helped to delineate the syndrome of PD (Lydiard and Ballenger, 1987). There are also studies shown that TCAs are effective in GAD (Rickels *et al.*, 1993) and OCD (Lydiard *et al.*, 1996).

- MAOIs, showed effectiveness in PD (Tiller *et al.*, 1997) and SAD (Liebowitz *et al.*, 1992), even in areas where BDZs were not very effective.

Serotonergic system

- SSRIs are effective across the board of anxiety disorders, and their favorable mile side-effect profile has elevated them to first-line treatment tools in these conditions.

- Buspirone, partial 5-HT_{1A} agonist, is effective in GAD (Rickels *et al.*, 1982)

Table 1 Efficacy of various classes of drugs in anxiety disorders

	PD	SAD	PTSD	GAD	OCD
BDZs	++	+	+	++	+
TCAs	++	+	+	++	++
MAOIs	++	++	+	+	?/-
SSRIs	++	++	+	+	++
Buspirone	-	?	?	++	?

PD, panic disorder; SAD, social anxiety disorder; PTSD, posttraumatic stress disorder; GAD, generalized anxiety disorder; OCD, obsessive-compulsive disorder; BDZs, benzodiazepines; TCAs, tricyclic antidepressants; MAOIs, monoamine oxidase inhibitors; SSRIs, selective serotonin reuptake inhibitors; Buspirone, partial 5-HT_{1A} agonist; +, some evidence; ++, firm evidence of efficacy; -, negative evidence; ?, discrepant evidence.

2.3 The neurochemistry of anxiety

Most works in the neurochemistry of anxiety have centered on the monoamines: serotonin and NE, and the $GABA_A$-BDZ receptor complex. Interest in the GABAergic, serotonergic, and noradrenergic systems originates from the clinical efficacy of compounds affecting them in the treatment of anxiety states and the study of their agonists and antagonists in the provocation of and prevention of anxiety. Within this complex neural network, neurochemical modulators may affect the activity within each brain area along the entire neurocircuitry system.

The prototypical full BDZ agonists, chlordiazepoxyde and diazepam, have anxiolytic properties and a wide safety margin. In experimental animals, anxiogenic situations modify the activity of ligands at BDZ sites, as do the ligands themselves (Clément *et al.*, 1997). To date the neurobiological mechanisms involved in modifications of behavioral and physiological reactions to extrinsic stimuli have not been discovered. Three main hypotheses are usually made (Malizia *et al.*, 1995). Firstly, anxiety is caused by the secretion of endogenous inverse agonists in the region of the BDZ receptor, decreasing GABAergic tone and therefore causing a decrease in inhibitory pathway function. Second, changes occur with the BDZ receptor and the activity of ligands is shifted towards the inverse agonist position in the spectrum. It may be hypothesized that patients with anxiety disorders have either differently

constituted BDZ-$GABA_A$ complexes, making them more vulnerable to anxious state, or that anxiety can induce such changes. Finally, an endogenous agonist tone can be observed in normal individuals; this is diminished or less effective in the presence of anxiety disorders.

There is evidence of involvement of the noradrenergic system in anxiety. Given that the central noradrenergic system modulates arousal, it has been hypothesized that increased noradrenergic activity leads to overarousal and anxiety. This was originally based on experimental work in animals suggesting that activation of the locus coeruleus produced fear reactions (Gray, 1982).

Because of the effectiveness of drugs acting on serotonergic system such as SSRIs and buspirone (partial 5-HT_{1A} agonist) in the treatment of anxiety disorders strongly implicate serotonin in anxiety. Therefore, further review emphasized on the serotonergic system in regulating anxiety.

B. Serotonergic system

1. Neuroanatomical organization of serotonergic neurons in brain

Anatomical investigations have revealed that serotonergic neurons arising from the brainstem raphe nuclei provide an extensive and widespread innervation of the cerebral cortex and key limbic structures (hippocampus, septum) (Figure 2). The two most important clusters of serotonergic cells are found in the dorsal and median raphe nuclei. Ascending serotonergic projections innervating the cerebral cortex and other regions of the forebrain arise primarily from the dorsal raphe and median raphe. The median raphe projects heavily to hippocampus, septum and hypothalamus, whereas the striatum is innervated predominantly by the dorsal raphe. Raphe neurons send collateral axons to areas of brain that are functional related, i.e. the amygdala and hippocampus or the substantia nigra and caudate putamen. Dorsal raphe axons appear to be more vulnerable to certain neurotoxic amphetamine derivatives, such as d-fenfluramine, 3,4-methylenedioxymethamphetamine (MDMA, commonly termed Ecstasy) or parachloroamphetamine (PCA). Median raphe axons appear to be more resistant to the neurotoxic effects of these drugs.

The raphe nuclei also receive input from other cell body groups in the brainstem, such as the substantia nigra and ventral tegmental area (DA), superior vestibular nucleus (acetylcholine; ACh), locus coeruleus (NE) and nucleus prepositus

hypoglossi and nucleus of the solitary tract (epinephrine). Other afferents include neurons from the hypothalamus, thalamus and limbic forebrain structures.

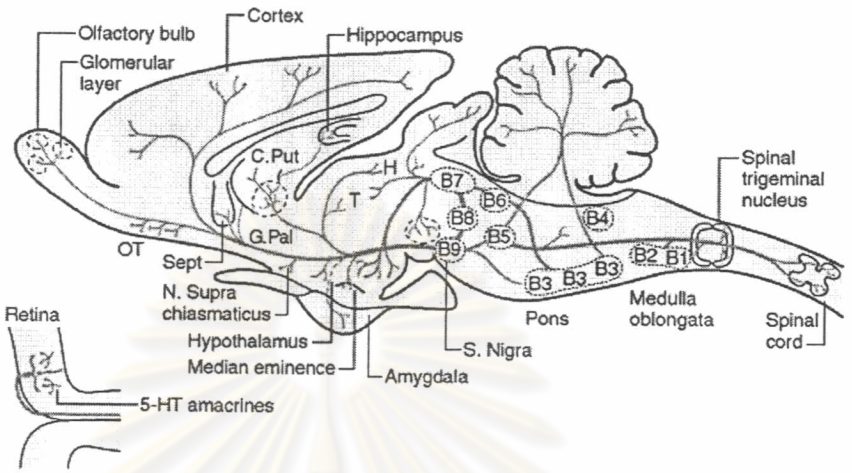

Figure 2. Schematic drawing depicting the location of the serotonergic cell body groups in a sagittal section of the rat central nervous system and their major projections. OT, olfactory tuberculum; Sept, septum; C. Put, nucleus caudate-putamen; G. Pal, globus pallidus; T, thalamus; H, habenula; S. Nigra, substantia nigra; B1-B9, groups of serotonin containing cell bodies (Frazer and Hensler, 1999).

2. Serotonin synthesis and control

Serotonin also called 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter. It is found throughout the body, with only a relatively small portion present in the brain. It cannot cross the blood-brain barrier and must be produced within the brain. The synthesis and primary metabolic pathway of 5-HT are shown in Figure 3. The initial step in the synthesis of 5-HT is the facilitated transport of the amino acid L-tryptophan from blood into brain. Serotonergic neurons contain the enzyme tryptophan hydroxylase (TPH), the rate limiting enzyme, which convert tryptophan to 5-hydroxytryptophan (5-HTP). Another enzyme involved in the 5-HT synthesis is aromatic L-amino acid decarboxylase (AADC) converting 5-HTP to 5-HT. Monoamine oxidase (MAO) converts 5-HT to 5-hydroxyindoleacetaldehyde, and this product is oxidized by an NAD^+-dependent aldehyde dehydrogenase to form 5-hydroxyindoleacetic acid (5-HIAA). In brain, 5-HIAA is the primary metabolite of 5-HT.

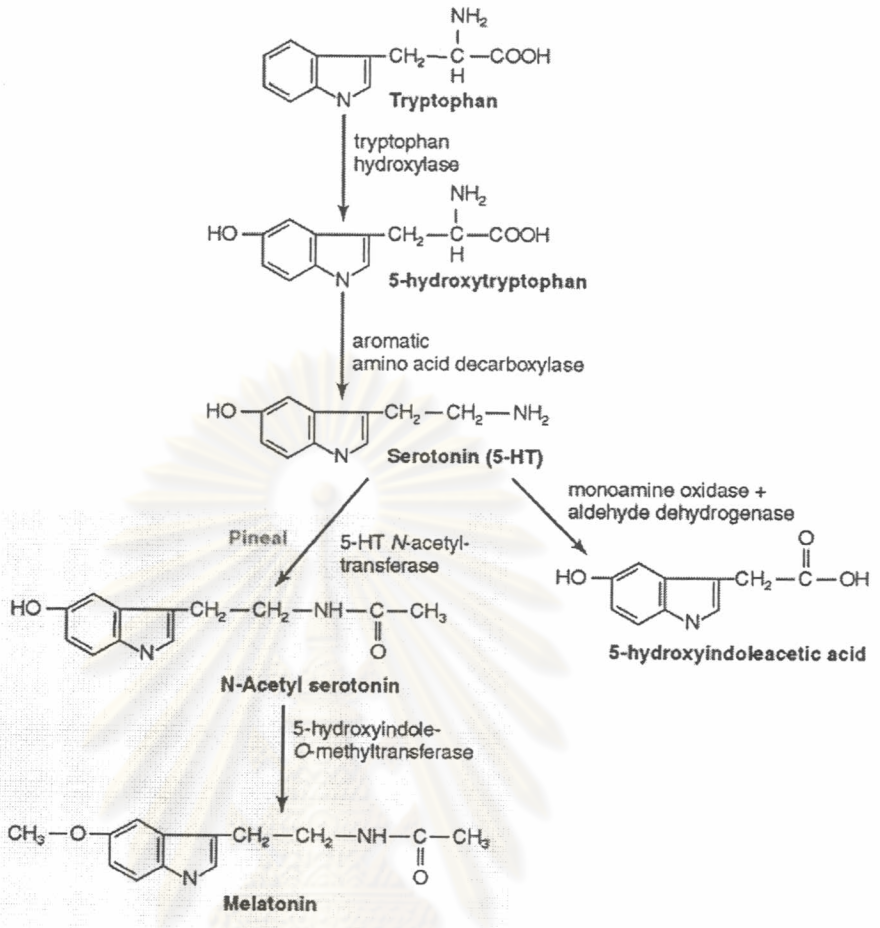

Figure 3. The biosynthesis and catabolism of 5-HT. Note that in the pineal gland, 5-HT is converted enzymatically to melatonin (Frazer and Hensler, 1999).

The activity of 5-HT in the synapse is terminated primarily by its re-uptake into serotonergic terminals. Synaptic effects of many amino acid and monoaminergic neurotransmitters, including 5-HT, are terminated by binding of these molecules to specific transporter proteins. The SERT is located on serotonergic neurons. Activity of the SERT regulates the concentration of 5-HT in the synapse, thereby influencing synaptic transmission.

3. Serotonin receptors

Investigators have identified at least nine different types of 5-HT receptors: $5\text{-HT}_{1A\text{-}1B}$, $5\text{-HT}_{1D\text{-}1F}$, $5\text{-HT}_{2A\text{-}2C}$, and 5-HT_3 (Frazer and Hensler, 1999). All 5-HT receptors are members of the G protein-coupled receptor superfamily except for the 5-HT_3 receptor, which is a ligand-gated ion channel.

3.1 5-HT_1 Receptor

The 5-HT_1 receptor family is negatively coupled to adenylyl cyclase. The 5-HT_{1A} receptor is coupled via G proteins to two distinct effector systems: (i) inhibition of adenylyl cyclase activity and (ii) the opening of K^+ channels, which results in neuronal hyperpolarization. In terminal field areas of serotonergic innervation, such as the hippocampus, 5-HT_{1A} receptors are coupled to both effector systems. However, in the dorsal raphe nucleus, 5-HT_{1A} receptors are coupled only to the opening of potassium channel. 5-HT_{1A} receptors are present in high density in the hippocampus, septum, amygdala, hypothalamus and neocortex. 5-HT_{1A} receptors are also present at high density in serotonergic cell body areas, in particular the dorsal and median raphe nuclei, where they function as somatodendritic autoreceptors, modulating the activity of serotonergic neurons. Activation of these autoreceptors causes a decrease in the firing rate of serotonergic neurons and a reduction in the release of 5-HT from serotonergic terminals.

The 5-HT_{1B} and 5-HT_{1D} receptor subtypes are also linked to inhibition of adenylyl cyclase activity. Binding sites that have been defined pharmacologically as 5-HT_{1B} receptors have been characterized in the rat, mouse and hamster, whereas the 5-HT_{1D} receptor has been characterized using pharmacological criteria in species such as guinea pig, pig, cow and human. The 5-HT_{1B} receptor in rats and mice and the 5-HT_{1D} receptor in bovine and human brain are located in high density in the basal ganglia, particularly in the globus pallidus and the substantia nigra. Functional studies indicate that the 5-HT_{1B} and 5-HT_{1D} receptors are located on presynaptic terminals of serotonergic neurons and modulate the release of 5-HT. Release of 5-HT from the dorsal raphe nucleus also appears to be under the control of 5-$HT_{1B/1D}$ receptors, although it is unclear whether these receptors are located on serotonergic terminals or cell bodies. The 5-HT_{1B} and 5-HT_{1D} receptors are also located postsynaptically, where they may modulate the release of other neurotransmitters, such as ACh in the hippocampus and DA in the prefrontal cortex.

The 5-HT_{1E} and 5-HT_{1F} receptor subtypes are coupled to the inhibition of adenylyl cyclase activity. 5-HT_{1E} receptor mRNA has been found in the caudate putamen, parietal cortex and olfactory tubercle (Lucas and Hen, 1995). The function of the 5-HT_{1E} receptor in intact tissue is not known due to the lack of selective agonists or antagonists. 5-HT_{1F} receptor mRNA is found in cortex, hippocampus,

dentate gyrus, nucleus of the solitary tract, spinal cord, trigeminal ganglion neurons, uterus and mesentery. Because selective agonists or antagonists for the 5-HT_{1F} receptor have also not been available until very recently, little is known about the distribution or function of the 5-HT_{1F} receptor in brain. Activation of 5-HT_{1F} receptors *in vivo* inhibits neurogenic dural inflammation and dural protein extravasation. (Wainscott, 1996).

3.2 5-HT_2 Receptor

The 5-HT_2 receptor family stimulates phosphoinositide-specific phospholipase C (PI-PLC). 5-HT_{2A} receptor-mediated stimulation of phospho-inositide hydrolysis has been well characterized in cerebral cortex. 5-HT_{2C} receptor-mediated stimulation of inositol lipid hydrolysis has been studied in the choroid plexus. Activation of 5-HT_{2A} receptors causes neuronal depolarization as a result of the closing of potassium channels. A high density of 5-HT_{2A} receptors is found in many cortical areas, particularly concentrated in the frontal cortex. 5-HT_{2A} receptors are also found in high density in the claustrum, a region connected to the visual cortex; in parts of the limbic system; and in the basal ganglia and the olfactory nuclei. Because of the lack of selective agonists to differentiate between members of the 5-HT_2 receptor family, many of the functional and clinical correlates of the 5-HT_{2A} receptor may very well involve or attribute to the 5-HT_{2C} receptor. 5-HT_{2C} receptors are present in high density in the choroid plexus. High-resolution autoradiography has shown that they are enriched on the epithelial cells of the choroid plexus. It has been proposed that 5-HT-induced activation of 5-HT_{2C} receptors could regulate the composition and volume of the cerebrospinal fluid. 5-HT_{2C} receptors are also found throughout the brain, particularly in areas of the limbic system, including the hypothalamus, hippocampus, septum, neocortex and regions associated with motor behavior, such as the substantia nigra and globus pallidus. However, in these areas, 5-HT_{2C} receptors are present in much lower concentrations than in the choroid plexus. The lack of truly selective 5-HT_{2C} receptor agonists and antagonists has limited our knowledge about the functional role of these receptors in brain.

The 5-HT_{2B} receptor was found low level in the brain as compared to peripheral tissues; however, it has been detected in the amygdala, lateral septum and hypothalamus (Duxon *et al.*, 1997).

3.3 5-HT_3 Receptor

The 5-HT_3 receptor is a 5-HT-gated cation channel that causes rapid depolarization of neuron. This depolarization is caused by a transient inward current, specifically the opening of a channel cation. 5-HT_3 receptors initially appeared to be confined to peripheral neurons, where they induce depolarization and therefore modulate neurotransmitter release. 5-HT_3 receptors are found in high density in peripheral ganglia and nerves, including the superior cervical ganglion and vagus nerve, as well as in the substantia gelatinosa of the spinal cord. 5-HT_3 receptors are located postsynaptically, where they modulate the release of other neurotransmitters such as ACh or DA. For example, in the ventral tegmental area, it modulates the activity of dopaminergic neurons, and in the cortex and hippocampus, it modulates GABAergic. The highest density of 5-HT_3 receptor sites in the brain is in the area postrema, the site of the chemoreceptor trigger zone.

The complex pharmacology and neuroanatomy of the serotonergic system is appropriate to allow this transmitter substance to broadly affect behavior and mood (Eison, 1989). Moreover, many commonly used antidepressant and antianxiety medications target this system (Lopez-Ibor, 1988). It is now cleared that altering serotonergic neurotransmission through pharmacological manipulation is a complex process involving presynaptic autoreceptors (5-$HT_{1A/1D}$), the SERT, and at least nine different postsynaptic receptor subtypes, of which several are believed to be potentially important in mood and anxiety (5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_3); (Hoyer *et al.*, 2002; Roth *et al.*, 2000).

4. Serotonergic system and anxiety

The changes in the serotonergic system have been strongly demonstrated to relate with anxiety disorders. Many clinical studies have indicated that various 5-HT-related agents, such as SSRIs, 5-HT_{1A} receptor agonists, 5-HT_2 receptor antagonists, MAOIs, and 5-HT-precursor, 5-hydroxy-L-tryptophan, are effective in the treatment of anxiety disorders (reviewed by Hashimoto *et al.*, 1999). Abnormalities in serotonergic function in anxiety may be the result of varied processes, including deficient or excessive innervations to key structures, and/or cellular mechanisms resulting in aberrant neurotransmission. At the cellular level, abnormalities may include abnormal regulation of 5-HT synthesis, release and/or

reuptake or abnormal responsiveness to 5-HT signal. In addition, the alteration of this neurotransmission also involved the presynaptic autoreceptors (5-HT_{1A}), the SERT site, and the postsynaptic receptors, of which several are believed potentially important in anxiety (5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}) (Figure 4).

4.1 5-HT_{1A} Receptor

In animal models, there was opposite effects of activation of 5-HT_{1A} receptor on anxiety with activation of its presynaptic receptors in the raphe nuclei resulting in anxiolysis, whereas activation of its postsynaptic receptors in the dorsal hippocampus resulting in an increase in anxious behaviors (DeVry, 1995; File, 1996; File and Gonzalez, 1996). The sum of the preclinical literature supports a primary role for presynaptic 5-HT_{1A} receptors in the anxiolytic effects of the 5-HT_{1A} agonists (DeVry, 1995). The results of 5-HT_{1A} receptor knock-out studies suggest that the overall behavioral outcome of loss of the 5-HT_{1A} receptor is an increase in anxiety-like behaviors (Ramboz *et al.*, 1998). They have used homologous recombination to generate mice lacking 5-HT_{1A} receptor subtype. They demonstrated that mice without 5-HT_{1A} receptors display less exploratory activity and increased fear of aversive environment (open and elevated spaces) of EPM. Gross and his coworkers (2002) used a tissue-specific, conditional rescue strategy to show that expression of the 5-HT_{1A} receptor primarily in the hippocampus and cortex, but not in the raphe nuclei, was sufficient to rescue the behavioral phenotype of the knockout mice. Furthermore, using the conditional nature of these transgenic mice, they suggested that receptor expression during the early postnatal period, but not in the adult, was necessary for this behavioral rescue. These findings showed that postnatal developmental processes help to establish adult anxiety-like behavior.

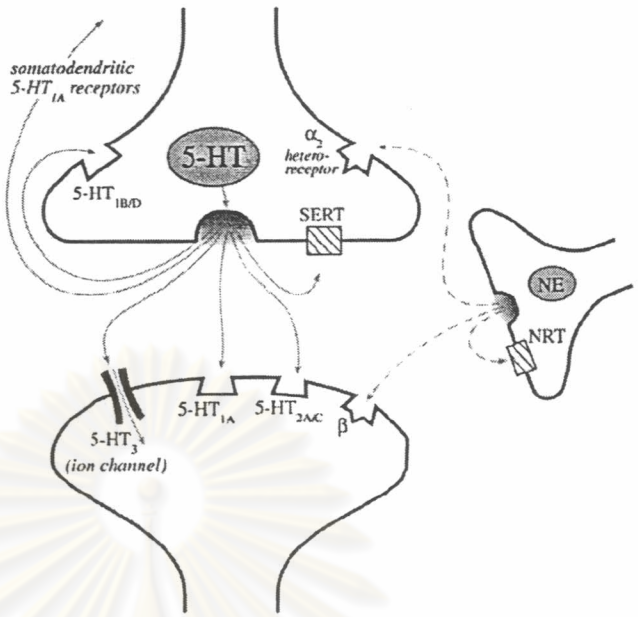

Figure 4. Targets within the 5-HT synapse. Release of 5-HT results in binding to several target receptors, with the resultant action being a summation of effects, including modulation by heteroreceptors, such as the α-2 and β-adrenergic receptors. SERT, 5-HT reuptake transporter; NRT, norepinephrine reuptake transporter; NE, norepinephrine (Kent *et al.*, 2002).

4.2 5-HT$_{1B}$ Receptor

A selective 5-HT$_{1B}$ receptor agonist, CP 94,253, has been determined to have significant anxiogenic effects in a rodent model, which were effectively blocked by pretreatment with a 5-HT$_{1B/1D}$ receptor antagonist (Lin and Parsons, 2002). A role of the 5-HT$_{1B}$ receptor in anxiety has been further supported by a knockout study that mice genetically lacking the 5-HT$_{1B}$ receptor showed less anxiety, consistent with the anxiogenic effects of the 5-HT$_{1B}$ receptor agonists (Mayorga *et al.*, 2001; Zhuang *et al.*, 1999).

4.3 5-HT$_{2A}$ Receptor

5-HT$_{2A}$ receptor antagonists have been demonstrated to be anxiolytic in various animal models, including conditioned fear model. Critchley and Handley (1987) showed that 5-HT$_{2A}$ receptor antagonists produced increasing in open/total arm entry ratio in the EPM model of anxiety at doses which did not affect total entries. In conditioned fear stress (CFS), 5-HT$_{2A}$ receptor antagonists significantly attenuated the CFS-induced freezing behavior (Ishida-Tokuda *et al.*, 1996).

4.4 5-HT_{2B} Receptor

The selective 5-HT_{2B} receptor agonist, VW 723C86 has been shown to be anxiolytic in a rodent social interaction test when injected directly into the medial amygdala. In addition, this effect could be prevented by pretreatment with the 5-$HT_{2C/2B}$ receptor antagonist (Duxon *et al.*, 1997).

4.5 5-HT_{2C} Receptor

The selective 5-HT_{2C} receptor antagonists, such as SB-242084, have been shown to be anxiolytic in animal models (Kennett *et al.*, 1997; Martin *et al.*, 2002). Kennett and colleagues (1997) demonstrated that SB-242084 exhibited an anxiolytic-like profile in the rat social interaction test by increasing time spent in social interaction. Further, treatment with SB-242084 demonstrated anxiolytic-like behaviors by increasing time spent, distance traveled, and entries into open arms in the EPM task (Martin *et al.*, 2002).

4.6 5-HT_3 Receptor

Successful anxiolysis has been reported with the 5-HT_3 receptor antagonists, ondansetron and MCI-225 in animal models (Eguchi *et al.*, 2001). In social interaction test in rats, these drugs significantly increased social interaction to unfamiliar partner without changes in ambulation. Moreover, in the EPM test in rats, MCI-225 increased the number of entries into the open arms, while ondansetron was less effective.

4.7 Serotonin Reuptake Transporter

In human, SERT is the primary target of the clinically successful class of antidepressant and antianxiety drugs known as the SSRIs. Although the putative antidepressant and antianxiety action of the SSRIs is generally understood to involve an increase in 5-HT concentrations in the synapse, resulting in increased postsynaptic receptor binding, this has not been clearly established. The SSRIs do block the reuptake of secreted 5-HT, preventing it from being transported back into the presynaptic neuron following discharge; however, a straightforward increase in the synaptic concentration of 5-HT may not be the end result, due to the presence of autoreceptors on the presynaptic neuron. These autoreceptors, which are located on

both the cell body (5-HT_{1A}) and the axon (5-HT_{1D}), regulate the release of 5-HT and therefore affect the net amount of 5-HT available in the synapse. Long-term administration of SSRIs has been shown to desensitize these 5-HT autoreceptors, thereby increasing the availability of extracellular 5-HT (Beasley *et al.*, 1992; Blier *et al.*, 1990). This may account for the latency in clinical efficacy, and provide support for an overall enhancement of serotonergic neurotransmission as the therapeutic mechanism of action of the SSRIs.

A seeming paradox exists in our current understanding of the role of 5-HT in anxiety. On the one hand is the preclinical observation that 5-HT facilitates avoidance and that antiserotonergic drugs have a role in reducing fear. This hypothesis is based on a significant body of animal literature demonstrating an increase in aversive behavior with increased 5-HT function and an anxiolytic effect of reducing serotonergic function (Chopin and Briley, 1987; Iverson, 1984). Even though, treatment of anxiety disorders with the SSRIs is very successful, SSRIs were indicated to increase in serotonergic tone and may cause anxiolytic in humans. In summary, the effect of the SSRIs on postsynaptic receptor regulation remains controversy. Furthermore, the antidepressant and antianxiety effects of the SSRIs may also involve other systems and mechanisms, including affecting the NE and neuropeptide systems (Szabo *et al.*, 2000).

C. Estrogen

1. Estrogen effects and its synthesis

Estrogens are steroid hormones that exert a wide range of effects throughout the body, including the central nervous system. Estrogens are required for normal female sexual maturation; they promote growth and differentiation of the breast, uterus, fallopian tubes, vagina, and ovaries (Carr, 1998). Moreover, estrogens are important for bone maintenance (Turner *et al.*, 1994) and have a protective role in the cardiovascular system (Farhat *et al.*, 1996). In the brain, estrogens appear to modulate the regulation of autonomic and reproductive neuroendocrine systems, mood, and cognition (Luine *et al.*, 1998; McEwen *et al.*, 1997; Pfaff, 1980).

In non-pregnant premenopausal women, estrogens are primarily synthesized in the ovaries, using cholesterol as a precursor (Figure 5). The most

potent and dominating estrogen in humans is 17β-estradiol, but also lower levels of the estrogens estrone and estriol are present. Following synthesis in the ovaries, most estradiol are bound to plasma proteins and transported to target tissues. The majority of the estradiol (60%) is bound to serum albumin, 38% is bound to sex hormone-binding globulin, and 2-3% is free (Carr, 1998). Steroid hormones are lipophilic and have a low molecular weight that enables them to diffuse freely through the cell membrane without the requirement of specialized transport systems. The steroid hormones are also believed to pass through the blood-brain-barrier via lipid-mediated, rather than carrier-mediated transportor (Pardridge, 1994). In both men and women, estrogens are also synthesized locally in non-endocrine tissues such as the brain, adipose tissue, and liver by the conversion of androgens to estrogens due to the presence of the aromatase cytochrome P450 enzyme (Figure 5). In the brain, this aromatase has been detected in several hypothalamic and limbic areas (Rees *et al.*, 1986; Horvarth *et al.*, 1997) and the locally produced estradiol has been shown to play an important role in neurogenesis and synaptogenesis during development (Naftolin *et al.*, 1988).

2. Genomic and nongenomic mechanisms of estrogen action

The presence of estrogen receptors (ERs) was first suggested by the work of Jensen and colleagues (Jensen and Jacobsen, 1962; Jensen and DeSombre, 1972). In the mid-1980s, the first ER cDNA was cloned (Walter *et al.*, 1985; Green *et al.*, 1986) and this receptor, presently known as ER_{α}. It was not until recently that a new ER subtype, ER_{β}, was discovered and cloned (Kuiper *et al.*, 1996; Mosselman *et al.*, 1996). The ERs belong to a large family of transcription factors, the nuclear receptor superfamily, that are intracellular and share many common properties such as nuclear localization and sequence specific DNA binding (Carson-Jurica *et al.*, 1990; Mangelsdorf *et al.*, 1995). The nuclear receptor proteins are composed of multiple functional domains and are characterized by the highly conserved DNA binding domain, which targets the receptor to specific DNA sequences known as hormone responsive elements (Chandler *et al.*, 1983; Klein-Hitpass *et al.*, 1986).

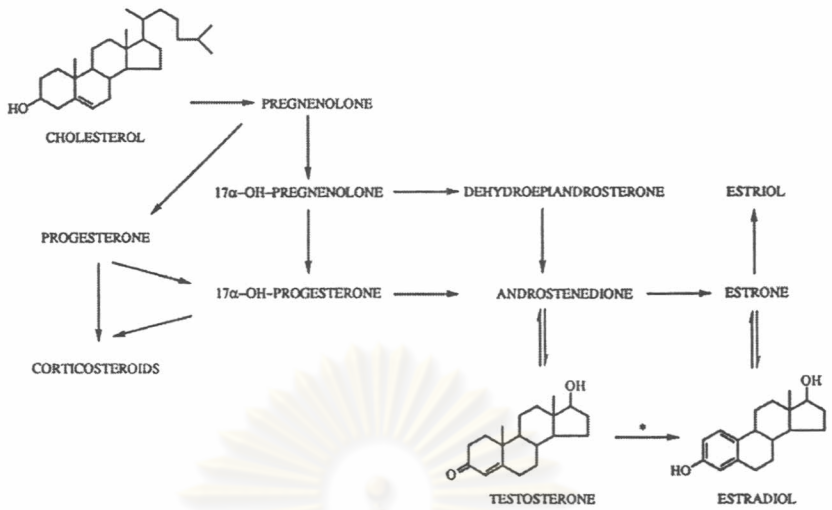

Figure 5. The biosynthetic pathway for the estrogens. Cholesterol is the main precursor and the enzyme aromatase cyotchrome P450 (*) is responsible for the conversion of testosterone to estradiol.

The genomic actions of estrogen are mediated via intracellular ER_α and ER_β receptors: following a conformational change, receptor-estrogen dimers bind to a palindromic DNA sequence which, together with several co-factors, leads to alterations in the gene transcription of estrogen-responsive elements (Driggers and Segars, 2002; Littleton-Kearney *et al.*, 2002). These genomic actions, which are exerted over a time-scale of hours or more, are responsible for the neurotrophic and neuroprotective actions of estrogen (Wise *et al.*, 2001; Vasudevan *et al.*, 2002). However, estrogen also elicits rapid (over seconds) changes in neuronal activity reflecting non-genomic actions. Though still poorly understood, they were found that the non-genomic actions of estrogen involved alterations in the activity of G-protein receptor-coupled transduction cascades, diverse protein kinases, adenylyl cyclase and nitric oxide (Driggers and Segars, 2002). They were also found their modulation of current flux through ion channels, and interactions with neurotrophins (Littleton-Kearney *et al.*, 2002). These actions which can evoke further downstream changes in cellular gene expression, at least partially, are mediated by membrane-localized receptors closely-related to intracellular ER_α and ER_β sites, though other mechanisms probably also intervene (Nadal *et al.*, 2000; Wise *et al.*, 2001). As an additional complication, it should be mentioned that ER receptors are susceptible to activation by phosphorylation, providing a potential mechanism for their estrogen-independent

recruitment by a variety of intracellular signals (Litttleton-Kearney *et al.*, 2002; Segars and Driggers, 2002).

3. Estrogen effects on the central nervous system

We have known for more than 40 years that estrogens target the brain of experimental animals. The first animal studies focused on estrogen actions on the hypothalamus affecting ovulation and reproductive behaviors. To date, both ER subtypes are predominantly found in limbic related areas of the brain such as the amygdala, hypothalamus, and septum (reviewed by Österlund and Hurd, 2001). Therefore, estrogens should exert many actions on brain areas that are important for learning, memory, emotions, and affective state as well as motor coordination and pain sensitivity (McEwen and Alves, 1999) (Table 2). Targets of estrogen action (McEwen and Alves, 1999), as summarized in Table 3, are many widely projecting neural systems such as the basal forebrain cholinergic system, the midbrain serotonergic and dopaminergic systems, and the brainstem cholinergic and noradrenergic systems.

4. Estrogen and anxiety

Sex discrepancy has been recognized in anxiety disorders, and more specifically most studies indicated that more women than men suffer from social anxiety disorder, specific phobias, PD associated to agoraphobia and GAD (reviewed by Palanza, 2001). There is some indication that women become more anxious during times of relatively low levels of circulating estrogen (Blank *et al.*, 1980). In addition, it is generally agreed that, in women, the incidence of these disorders dramatically increases after puberty (Earls, 1987), and it is greater during women's reproductive lives (Bebbington *et al.*, 1998). These differences suggest the importance of reproductive hormones and cyclical hormonal patterns in the prevalence of anxiety disorders in women. Additionally, the menopausal women show symptoms of cognitive dysfunction, depression and anxiety (Arpels, 1996; Campbell and Whitehead, 1977; Sherwin, 1998) which were improved with estrogen replacement therapy (ERT) (Halbreich, 1997; Sherwin, 1998). These findings implicate the pivotal role of estrogen in controlling emotion, especially anxiety levels in women.

Table 2 Estrogen effects on clinically relevant non-reproductive functions

Affective state and mood	Estrogens affect the serotonergic, noradrenergic, dopaminergic, and cholinergic systems, all of which play a role in affective state and mood. Two disorders are particularly noteworthy, premenstrual syndrome (PMS) and depressive illness. For PMS, suppression of ovarian cyclicity reduces mood swings, although specific hormonal mechanisms are not known. High doses of estrogens have antidepressant effects in human subjects, and estrogen treatment influences the response to antidepressant drugs in animal models.
Cognitive function	Estrogens influence short-term verbal memory as well as performance on tests of fine motor skills and spatial ability. Sex differences exist in humans and in animals for strategies used in solving spatial navigation problems.
Dementia	Estrogen therapy in open trials has been reported to prospectively benefit cognitive function in nondemented women. There is a reportedly lower prevalance of Alzheimer's disease as a cause of death in elderly women who receive ERT postmenopausally.
Motor coordination and movement disorders	Estrogens modulate activity of the cerebellum and the nigrostriatal and mesolimbic dopaminergic systems and have effects on normal and abnormal locomotor activity. High levels of estrogens antagonize the dopaminergic system and are recognized to exacerbate symptoms of Parkinson's disease, whereas low estrogen levels facilitate dopaminergic function.
Excitability and epilepsy	Catamenial epilepsy varies according to the menstrual cycle, with a peak frequency corresponding to the lowest ratio of progesterone to estradiol during the cycle. Three potential mechanisms are recognized: (1) estrogen induction of excitatory synapses in hippocampus, leading to decreased seizure thresholds; (2) progesterone actions via the steroid metabolites which act via the GABAa receptor to decrease excitability; (3) hormone actions on the liver to increase clearance rates of antiseizure medication.
Pain	Recent studies in mice indicate that males and females use functionally distinct pain pathways, and that gonadal steroids, particularly estrogens, play a major role in regulating these pathways.
Stroke	Estrogens protect against damage produced by ischemia in experimental models of stroke.

(McEwen and Alves, 1999)

Table 3 Brain regions affected by estrogens

Basal forebrain cholinergic system	Estradiol treatment up-regulates cholinergic markers and nerve growth factor receptors, promoting neuronal survival; there are sex differences programmed during early development.
Midbrain serotonergic system	Estrogen treatment regulates tryptophan hydroxylase, SERT, and certain 5-HT receptor subtypes; there are sex differences in progestin receptor expression and in 5-HT turnover.
Midbrain and hypothalamic dopaminergic system and projections	Incertohypothalamic dopaminergic neurons show developmentally programmed sex differences in neuron number and function and respond to prolactin and estrogen treatment. In contrast, nigrostriatal and mesolimbic dopaminergic neurons fail to express detectable intracellular ER; however, estrogen facilitates amphetamine- or apomorphine-stimulated DA release and locomotor activity in rats.
Brainstem catecholaminergic systems	Estradiol regulates tyrosine hydroxylase gene and immediate early gene expression, and does so apparently via intracellular ERs.
Hippocampus	Estrogen treatment induces de novo synapse formation on pyramidal neurons, involving the participation of NMDA receptors.
Spinal cord	There are sex differences and estrogen modulation of nociception in humans and animals.
Glial cells	Estradiol regulates specific genes such as glial fibrillary acidic protein and apolipoprotein E within astrocytes and microglia via intracellular ERs, and these changes may reflect a role of glial cells in normal synaptic plasticity as well as lesion-induced plasticity.
Cerebral vasculature	Some intracellular ERs are expressed in central nervous system endothelia, and estrogen treatment regulates glucose utilization, possibly by inducing glucose transporter 1 in endothelial cells of the blood-brain barrier.

(McEwen and Alves, 1999)

The effect of estrogen on the anxiety-like behaviors has been studied in various animal models. In rodents, nonreproductive behaviors related to anxiety have been affected by changes in estrogen level, but the results are controversial. Studies looking at open arm exploration in the EPM indicated that cycling female rats are more active and less anxious than males (Leret *et al.*, 1994) and those estradiol benzoate-treated Ovx females spent a greater percentage of the time in the open arms than did their vehicle-treated counterparts (Nomikos and Spyraki, 1988). However, Blanchard and associates (1991) have presented evidence to support the contention that female rats are more anxious than male rats in response to potential dangers (cat and cat odor) presented in an anxiety/defense test battery. Johnston and File (1991) demonstrated a gender difference in three animal models of anxiety, notably EPM, modified Vogel conflict tests, and the social interaction; however, the results varied across three tests. The fluctuations of ovarian hormones in estrous cycle may relate with the inconsistency of anxiety-like behavior of the female animals. Serum estrogen corresponded with different phases of the cycle in that the levels during proestrus were higher when compared to those for estrus, metestrus, and diestrus. Reductions in behavioral indices of anxiety have been reported during the phases of proestrus as compared with diestrus (Marcondes *et al.*, 2001). The results suggested the responding to anxiety varied according to the estrous cycle. Treating diestrous female rats with 17β-estradiol can abolish the cycling difference (Marcondes *et al.*, 2001). It thus seemed likely that 17β-estradiol might produce the anxiolytic response during the estrous cycle of the rats. However, the mechanisms of estrogen in the controlling anxiety are not yet fully understood.

5. Estrogen effects on the serotonergic system

The effects of estrogen on the serotonergic nervous system of mammals are established (Maggi and Perez, 1985). Higher levels of 5-HT and 5-HIAA were found in the brains of female rats than in the brains of male rats (Carlsson *et al.*, 1985). In addition, the 5-HT synthetic capacity is higher in the female rat brain (Carlsson *et al.*, 1985; Dickinson and Curzon, 1986). Neurochemical and electrophysiological studies have shown modifications related to the estrous cycle in the activity of 5-HT brain systems (Uphouse *et al.*, 1986). During the estrous cycle of

the rat, there are changes in 5-HT binding (Biegon *et al.*, 1980); cortical [^{3}H]5-HT binding is low in the morning of proestrus and then increases during estrus (Uphouse *et al.*, 1986; Williams and Uphouse, 1989). Sumner and Fink (1997) found that 5-HT_{2A} binding sites were significantly increased at proestrus compared with diestrus in frontal and cingulate cortex, olfactory tubercle and nucleus accumbens.

In most studies of many species, ER_β is generally expressed in the dorsal raphe. In monkeys, ER_β is localized in serotonergic neurons at the mRNA and protein level (Bethea, 1994). The predominant isoform in the mouse raphe is ER_β, although ER_α is also present (Mitra *et al.*, 2001). ER_β is expressed in the dorsal raphe in guinea pigs and rats, suggesting that it is probably in serotonergic neurons as well (Lu *et al.*, 1999; Shughrue *et al.*, 1997). Therefore, ER_β appears to be the primary mediator of estrogen action within serotonergic neurons. There are differences between species in the expression of ER_α in serotonergic neurons. ER_α is neither found in the macaque serotonergic neurons nor in the rest of the dorsal raphe nuclei. In rats, ER_α was also not found in serotonergic neurons, but in smaller interneurons of the rat dorsal raphe (Alves *et al.*, 1998). In guinea pigs, ER_α has been detected in nonserotonergic neurons of the PAG region, adjacent to the raphe region (Lu *et al.*, 1999; Turcotte and Blaustein, 1993). The mouse raphe contains ER_α expressing neurons of serotonergic and nonserotonergic phenotypes.

Many experiments represented the effects of estrogens on the regulation of the components of the serotonergic system. Bethea and her coworkers (2000) previously demonstrated that estradiol and progesterone increase TPH mRNA levels in the dorsal raphe of macaques. In midbrain raphe of primates, estrogen treatment decreased SERT mRNA expression (Pecins-Thompson *et al.*, 1998). In macaque hypothalamus, estrogen treatment decreased expression of the $5HT_{2C}$ receptor in a number of hypothalamic nuclei (Gundlah *et al.*, 1999). In rat brain, after 32-h of estrogen treatment increased levels of $5HT_{2A}$ mRNA in dorsal raphe and $5HT_{2A}$ receptor binding in frontal, cingulate, and primary olfactory cortex as well as in nucleus accumbens (Sumner and Fink, 1995). There were no sex differences in this induction (Sumner and Fink, 1995). Another study on rats reported that after 24-h of estrogen treatment increased $5HT_{2A}$ mRNA levels in amygdala, hippocampus, nucleus accumbens, and a number of cortical areas, but decreased $5HT_{1A}$ mRNA

levels in many of the same brain regions (Österlund *et al.*, 1999). Chronic (2 wk) estrogen treatment also decreased $5HT_{1A}$ receptor binding in amygdala, hippocampus, and cerebral cortex (Österlund *et al.*, 2000). In this study, however, the effects of estrogen on $5HT_{1A}$ mRNA levels, evident after acute estrogen treatment, disappeared with the chronic treatment that decreased $5HT_{1A}$ receptor binding. Recently also demonstrated that Ovx rats have a supersensitivity of 5-HT_{1A} receptors and are prevented by estradiol (Raap *et al.*, 2002). The actions of estrogen on the $5HT_{1A}$ receptor system illustrate the complexities of distinguishing between traditional genomic effects of estrogens and those involving a nonnuclear action. Estrogen treatment causes a rapid decrease in coupling to G proteins that reduce the inhibitory effect of $5HT_{1A}$ agonists on lordosis behavior, hyperphagia, and oxytocin and adrenocorticotropic hormone responses (Mize *et al.*, 2001; Raap *et al.*, 2000). Regarding the rapid estrogen-induced decrease in $5HT_{1A}$ efficacy, this has been assessed by measuring binding of radiolabeled guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS) binding (Mize *et al.*, 2001) after treatment with estrogen in homogenates of hippocampus and frontal cortex. 17β-estradiol (EC_{50} = 25 nM) showed a dose-dependent ability to decrease GTPγS binding, and this effect was mimicked by diethylstilbestrol but not by the less potent estrogens, 17α-estradiol and estriol, and was blocked by the estrogen antagonist, ICI-182780 (Mize *et al.*, 2001). These results are consistent with the involvement of a nonnuclear form of ER_{α} or ER_{β}.

However, few studies have looked at the impact of estrogen on serotonergic system related to anxiety behavior.

D. Experimental models of anxiety

Animal tests of anxiety can be divided into several categories. These include those based on conditioned fear or conflict and those in which anxiety is generated by novel environments.

Conflict tests have been widely used (Pollard and Howard, 1989; Treit, 1985; Vogel *et al.*, 1971). The paradigm involves repeated alternating exposure to two different experimental schedules. In the Geller-Seifter test, rats are trained to press a lever to obtain a food reward (Treit, 1985). In the punished schedule the rats also receive an electric shock signaled by a light; whereas, in the unpunished schedule

pressing for the food reward is not punished by electric shock (Pollard and Howard, 1989). A similar protocol exists in the Vogel test but in this case thirsty rats are trained to drink from a spout (Vogel *et al.*, 1971). In both tests, it is presumed that the behavioral suppression (reduced response rate) that occurs in the punished "conflict" component of the schedule is a result of the animal anticipating the punishment. Anxiolytic drugs such as the BDZs increase rates of punished but not unpunished responding.

In contrast to conflict tests, tests involving exposure to novel environments do not depend on the rat being conditioned to fear a stimulus that is linked to a primary drive behavior such as eating or drinking. Instead, these ethologically based tests rely on the rodent innate fear of novel environments and measure natural behaviors. The EPM, the ETM, and the open field are the examples.

1. Elevated plus-maze

In the field of anxiety, the EPM has become one of the most popular animal models (Pellow *et al.*, 1985). Experiments carried out almost half a century ago, investigating approach-avoidance conflict (Montgomery, 1958), prompted the development of an apparatus in which the animal is given a choice between exploring a potentially threatening environment, an exposed runway, about 50 cm above the ground, or spend time in a relatively safe enclosure. The EPM in fact offers the animal two safe and two unsafe environments to explore, since it consists of four arms, arranged in a cross.

Using the EPM to measure anxiety is relatively simple: an anxious animal is one that chooses to spend time in the closed arms and rarely ventures out onto the open arms. One possible complication that an animal might not come out since it is inherently inactive, rather than anxious, can be dealt with by expressing the number of entries into the open arms as a percentage of the total arm entries. There are more sophisticated measurements, such as comparing the occurrence of different behaviors (grooming or rearing) and postures (the stretch-attend for example) in the two types of arm, but no one has shown that they give any better indication of anxiety than combinations of arm entries, time spent in each arm and distance traveled, all of which can be collected easily and reliably with video-tracking systems (Rodgers and Cole, 1994; Rodgers and Johnson, 1995; Rodgers *et al.*, 1997). Anxiolytic

compounds increase, whereas anxiogenic compounds decrease the percentage of time spent on open arms relative to total time on the maze (Cole *et al.*, 1995; Pellow *et al.*, 1985). The total number of entries and/or the number of entries into the closed arms reflects a measure of locomotor activity (Korte *et al.*, 1999), while antidepressants (imipramine, miansrin) and antipsychotics (haloperidol) do not (Pellow *et al.*, 1985; Pellow and File, 1986).

2. Elevated T-maze

While the EPM is a mixed model in the sense that multiple defense reactions are displayed while the rat freely explores the apparatus. The ETM is developed to discriminate, in the same rat, conditioned from unconditioned fear, which have been related to GAD and PD, respectively. It is derived from the widely used EPM (Pellow *et al.*, 1985) by sealing the entrance to one of the enclosed arms. In the experimental session, the rat performs two consecutive tasks, inhibitory avoidance and one-way escape, representing the conditioned and unconditioned fear, respectively. ETM consists of three arms of equal dimension, one enclosed arm positioned perpendicularly to the two opposed open arms and it is elevated. When placed at the end of the enclosed arm, the rat does not see the open arms until it pokes its head beyond the walls of the closed arm. To be on an open arm seems to be an aversive experience, since rats have an innate fear of height and openness (Montgomery, 1958; Pellow *et al.*, 1985, Treit *et al.*, 1993). This would allow the animal to learn "inhibitory avoidance" if repeatedly placed inside the enclosed arm to explore the maze. On the other hand, when the rat is placed at the end of the open arms it can move towards the closed arm, presumably performing an "escape response". Concerning inhibitory avoidance, the pharmacological results obtained so far show that this task was impaired by anxiolytic, BDZ (Graeff *et al.*, 1993; Viana *et al.*, 1994), 5-HT_{1A} agonists (Viana *et al.*, 1994), 5-HT_2 antagonist as well as specific 5-$HT_{2B/2C}$ antagonist (Mora *et al.*, 1997), and facilitated by anxiogenic agents, α_2-adrenoreceptor antagonist, and 5-$HT_{2B/2C}$ agonists (Mora *et al.*, 1997). In contrast, MAOIs, phenylthylamine hallucinogens, and one neuroleptic were ineffective (Mora *et al.*, 1997). Therefore, inhibitory avoidance seems to be selectively sensitive to drugs that change anxiety in human beings and/or other animal experimental models.

Since this task was impaired by the drugs, a BDZ and two azaspirones, which are known to improve GAD, inhibitory avoidance in the ETM may be a useful model for this psychiatric disorder. Deakin and Graeff (1991) have suggested that 5-HT facilitates conditioned fear (related to GAD) through stimulation of postsynaptic receptors located in prosencephalic regions innervated by 5-HT fibers coming from dorsal raphe nucleus. Consistent with this hypothesis, the injection of the excitatory amino acid kainic acid and the BDZ inverse agonist into the dorsal raphe nucleus facilitated inhibitory avoidance, which are supposed to stimulate serotonergic neurons when microinjected (Graeff *et al.*, 1996). Conversely, inhibition of serotonergic neurons by intra-dorsal raphe injection of the 5-HT_{1A} receptor stimulant impaired inhibitory avoidance in the ETM (Zangrossi *et al.*, 1999). This evidence further supported the correlation between inhibitory avoidance and GAD. In contrast to the inhibitory avoidance task, one-way escape from the open arm of the ETM proved sensitive to the 5-HT releaser and uptake inhibitor D-fenfluramine increased escape latencies in a dose-dependent manner. From Solyom (1994) had reported that D,L-fenfluramine has therapeutic effects in PD patients. All of these are consistent with the view that 5-HT inhibits unconditioned fear, related to PD, in the PAG (Deakin and Graeff, 1991). In conclusion, the pharmacological studies and reported clinical evidence indicated that the inhibitory avoidance task in the ETM may be related to GAD while the escape task may relate to PD.

3. Open field test

The open field test is now one of the most popular procedures in animal psychology (reviewed by Prut and Belzung, 2003). Different versions are available, differing in shape of the environment, lighting, presence of objects within the arena. The procedure generally usually involves forced confrontation of a rodent with the situation. The animal was placed in the center or close to the walls of the apparatus and the following behavioral parameters were recorded for a period ranging from 2 to 20 min (usually 5 min): horizontal locomotion (number of crossings of the lines marked on the floor), frequency of rearing (sometimes termed vertical activity), grooming (protracted washing of the coat). In such a situation, rodents spontaneously prefer the periphery of the apparatus more than the central parts of the open field. Indeed, mice and rats walk close to the walls, a behavior called thigmotaxis.

Increases of time spent in the central part as well as of the ratio central/total locomotion or increase of the latency to enter the central part are indications of anxiolysis.

The open field has become a convenient procedure to measure not only anxiety-like behaviors, but also sedation or activity. In fact, anxiety behavior in the open field is triggered by two factors: individual testing (the animal is separated from its social group) and agoraphobia (as the arena is very large relative to the animal's breeding or natural environment). It is clear that these two factors may trigger anxiety behavior only in gregarious species and/or in species that show fear of open spaces into which they are forced. This is precisely the case with rodents that live in social groups and in small tunnels. This is of course not the case in species such as lambs or cows that live in large fields. The effects of many different drugs have been investigated in the open field, including compounds with effective or potential anxiolytic effects (BDZs, 5-HT ligands, neuropeptides) but also compounds with stimulant (amphetamine, cocaine), sedative (neuroleptic) or prostration-inducing (epileptogenic drugs) activity. An increase in central locomotion or in time spent in the central part of the device without modification of total locomotion and of vertical exploration can be interpreted as an anxiolytic-like effect while the contrary, that is a decrease of these variables, is associated with anxiogenic effects. Increased locomotion can be considered a stimulant effect while decreased vertical activity and locomotion are related to sedation.