GABAERGIC MECHANISMS UNDERLYING ANXIETY BEHAVIOR IN THE POSTPARTUM RAT

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ABSTRACT

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Postpartum female rodents are less anxious than diestrous virgins in a number of behavioral paradigms. However, reproductive state effects on anxiety have rarely been studied in the light-dark box, and previous studies have been inconsistent. Therefore, I have readdressed the usefulness of the light-dark box to assess anxiety differences between postpartum and virgin female rats. Postpartum females did exhibit less anxiety behavior in a light-dark box then diestrous virgins, but only when ambient illumination was high. Furthermore, reduced postpartum anxiety in the light-dark box was dependent on both infant contact and $GABA_A$ receptor activity. Separating postpartum females from their pups for the four hours prior to light-dark box testing increased their anxiety to the level of diestrous virgins, and inhibiting the $GABA_A$ receptor at three different binding sites by using (+)-bicuculline (GABA site), FG-7142 (benzodiazepine site), or pentylenetetrazol (picrotoxin site) revealed that pentylenetetrazol produced the strongest anxiogenic effects, often specifically in postpartum and not diestrous virgin females. To investigate whether natural differences in binding to the $GABA_A$ receptor contributes to reduced postpartum anxiety, I used autoradiography assays to analyze binding to the picrotoxin ([^{35}TBPS], benzodiazepine ([^{3}H]FNP), and GABA's site ([^{3}H]MUSC]) on the receptor. I investigated six different neural sites where GABA is known to mediate anxiety behavior (mPFC, BST, CeA, Hipp, rPAG, cPAG) in the brains of early postpartum, mid-

pregnant, diestrous virgin and sexually naïve male rats to look at reproductive state and sex differences in binding. The only significant difference across my groups in binding in any brain region examined was higher [^{3}H]FNP binding in the brains of diestrous virgins in the DG and CA1 regions of the hippocampus in comparison to virgin males and mid-pregnant females, with no differences compared to postpartum females. Lastly, I used Western blot analysis to determine whether these four groups differed in concentration of subtypes of the alpha subunit, as the picrotoxin binding site is associated with this subunit. I examined content of the α2, α3, and α4 in the same six brain regions used previously and found that male rats had higher content of α2 subunit in the rPAG than did diestrous or postpartum females, and there were otherwise no differences in content of any of the subunits across groups in any brain regions investigated. Results from these three experimental chapters are discussed in relation to reduced anxiety behavior in postpartum rats and to the role that the picrotoxin binding site may play in postpartum anxiety.

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Introduction

"Emotion" is as an affective state of consciousness in which joy, sorrow, fear, hate or like is experienced and typically accompanied by appropriate physiological and behavioral responses. Emotional regulation, while a vast concept, can be considered as a reaction to relevant stimuli in the environment (Belzung & Philippot, 2007; Cacioppo et al., 1998). These relevant stimuli activate physiological systems, and thereby elicit the physical sensations associated with different emotions (Belzung & Philippot, 2007; Cacioppo et al., 1998; Lang, 1985). One emotion that is present in most psychopathological conditions is anxiety (Belzung & Philippot, 2007; Lang, 1985), an emotion that involves psychological, physiological and behavioral responses to a perceived threat or stressful stimulus (Belzung & Philippot, 2007; Bremner, 2004; Lang, 1985). In general, anxiety reactions are acute and often involve activation of the autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis to increase heart rate, blood pressure, respiration, and arousal (Belzung & Philippot, 2007; McEwen, 2000). These responses are adaptive and necessary for survival, because animals (including humans) need to mobilize body systems for action when presented with a dangerous stimulus and effectively respond to threats (McEwen, 2000; McEwen, 2007). Normal anxiety can be characterized as a repertoire of defensive reactions that allow an animal to meet threats (Rodgers, 1997). However, this acute response is sometimes not inhibited after the danger has passed, and an animal can experience inappropriate chronic anxiety instead of the normally adaptive response (Belzung & Philippot, 2007; McEwen, 2000; McEwen, 2007; Rodgers, 1997), which in humans can lead to anxiety disorders. An estimated 11% of people in the United States suffer from an anxiety disorder and another 8% have an anxiety disorder and at least one other additional psychiatric disorder (Greenberg et al., 1999). Anxiety disorders carry a high

economic burden, with an estimated cost of $42.3 billion in 1990 in this country (Greenberg et al., 1999), and are also extremely mentally, socially, and physically debilitating for those who suffer from these disorders (Ressler & Mayberg, 2007).

In both humans and non-human animals, chronic anxiety can cause many detrimental effects including elevated stress hormones, increase negative mood, memory deficits, gastrointestinal malfunction, decreased immune function, and sleep problems (De Quervain et al., 1998; Gareau et al., 2008; Herman et al., 2003; Howell & Muglia, 2006; McEwen, 2000; McEwen, 2007; Miller & O'Callaghan, 2003; Nichols et al., 2001; Touyarot et al., 2004). Of particular concern are the effects of anxiety during pregnancy and lactation in women. Chronic anxiety and stress during pregnancy leads to damaging effects on fetuses (McEwen, 2007; Meaney et al., 2007; Weaver et al., 2004), involving decreased uterine blood flow to the fetus, which could partially contribute to smaller birth weights of these infants (Glover, 1999). Lower birth weight is associated with health problems such as coronary heart disease later in life (Barker, 1995). In addition, chronic anxiety in women during the postpartum period can result in lower emotional attachment between mothers and infants (Adam et al., 2004; Barnett & Parker, 1986; Manassis et al., 1994; Zelkowitz & Papageorgiou, 2005), mothers abusing their infants (De Bellis et al., 2001; Maestripieri, 1999; Nayak & Milner, 1998), slower growth of infants (Barnett & Parker, 1986; O'Brien et al., 2004), and increased risk for higher anxiety in their offspring as adults (Barnett et al., 1991; Hirshfield et al., 1997; O'Connor et al., 2002).

Anxiety versus Fear

The terms "anxiety" and "fear" are often used interchangeably even though it has often been argued that they do not describe the same phenomenon (Davis et al., 1997; Grillon, 2008),

and there is a great deal of controversy over what distinguishes them. Cross-culturally, fear and anxiety are often considered to be virtually independent concepts. In many languages, fear is more closely connected with concepts like passion, anger, and violence, while anxiety is more often associated with restriction, restlessness, worry, and panic (Sartorius et al., 1990). The issue is further complicated because, while the terms appear to be used for different emotions originating from distinct sources, both feelings of fear and those of anxiety elicit similar activation of the autonomic nervous system and the HPA axis (Belzung & Philippot, 2007), making it more difficult to distinguish between the two even from a physiological standpoint. However, even considering these difficulties, there have been many attempts at defining these closely related phenomena.

Goodwin (1986) defines anxiety as an emotion that signifies the presence of an unidentified danger, or an emotion that is not proportional in intensity to the actual threat. Furthermore, he states that fear is different from anxiety in that fear signifies the presence of a known danger and that the strength of fear is proportionate to the degree of danger. More recent support for this distinction states that fear is an adaptive, protective emotion that allows an individual to evade or confront an identifiable threat, and which then subsides shortly after its onset (Belzung & Philippot, 2007; Bremner, 2004; Davis et al., 1997; Lang et al., 2000). Conversely, anxiety may appear physiologically similar to fear, but lacks adaptive significance because it is not in response to a clear stimulus and may last for long periods of time (Amstradter, 2008; Belzung & Philippot, 2007; Bremner, 2004; Davis et al., 1997; Lang et al., 2000). Furthermore, while fear is in response to an identified danger, anxiety can be a response to the anticipation of threat due to the presence of general cues that often signify danger, but that are not themselves dangerous (Amstradter, 2008; Barlow, 2002; Belzung & Philippot, 2007;

Bremner, 2004; Jelen et al., 2003; Lang et al., 2000). Additionally, there have been animal studies indicating that these two concepts are indeed independent phenomena, as rats selectively bred for high and low anxiety do not show consistent behavior in some anxiety paradigms versus some behavior tests that might be more related to fear (Yilmazer-Hanke et al., 2004). Specifically, these "high anxiety-related behavior" rats show higher anxiety than "low anxiety-related behavior" rats in an elevated plus-maze (Neumann, 2003; Yilmazer-Handke et al., 2004), but the two lines do not differ in their fear responses in the fear-sensitized acoustic startle paradigm (Yilmazer-Handke et al., 2004). It has even been suggested that the regulation of fear and anxiety can be separated and segregated to different neural regions in some cases, and that specific brain regions may have large regulatory roles on behavioral effects in paradigms testing either fear or anxiety, but not in paradigms testing the other (Davis & Shi, 1999). Thus, for present purposes, fear can be best understood as a situation-appropriate, acute response to an explicit threat, while anxiety is a longer-lasting response that is either anticipatory or elicited in the absence of an explicit threat.

Assessing Anxiety in Humans and Laboratory Animals

A tremendous amount of work has been done to try to understand human anxiety and its specific variants, such as panic, phobia, generalized anxiety, obsessive-compulsive, and post-traumatic stress disorder (Amstadter, 2008; Barlow, 2002; Brown & Barlow, 2002; McIntyre et al., 2006; Rauch et al., 1997; Ressler & Mayberg, 2007). Researchers use a variety of methods to study human anxiety, including questionnaires, physiological measurement, blinking startle response, biological challenge, studying patients with brain damage, comparing patients with anxiety disorders to healthy controls, electroencephalogram measures of brain activity, and

neuroimaging (Amstadter, 2008; Bremner, 2004; Davidson, 2002; Graeff et al., 2003; Hadjikhani et al., 2003; LeDoux, 2000; Murphy et al., 2003; Phan et al., 2002; Rauch et al., 1997; Schmidt & Richey, 2008; Von Bardeleben & Holsboer, 1988). When used in combination, these techniques can be very helpful in studying and understanding human anxiety (Amstadter, 2008; Davidson, 2002; Davidson, 2000b). For example, researchers can compare the brain activity of patients with anxiety disorders to healthy controls during a stressful situation, which can identify brain regions that are differentially activated, indicating that those regions could be involved in anxiety (Amstadter, 2008; Davidson, 2002; Davidson, 2000b). Through a combination of such techniques, researchers have and continue to make novel discoveries about human anxiety and move towards better more efficient treatments of anxiety disorders (Amstadter, 2008). Of course, there are crucial studies that cannot ethically be conducted using human subjects, so for some questions we must turn to animal models to study anxiety (Bourin et al., 2007). Because animals cannot use verbal cues to express their anxiety state as humans can, indirect measures must be used to study their anxiety. Observable responses that can indicate anxiety in animals include freezing, escape behavior, increased startle, increased heart rate and blood pressure, greater sensitivity to pain, and elevated stress hormones (Belzung & Philippot, 2007; Grillon, 2008; Lang, 2000; Millan, 2003). Many different behavioral paradigms have been created so that researchers can assess animals' anxiety state through observing their behavior in somewhat standardized paradigms, and many of these paradigms are used as screening tools for possible treatments in humans (Chadman et al., 2009; Rodgers, 1997).

There are many different types of unconditioned tests used to study anxiety behavior in animals. Examples include conspecific interaction tests (such as social interaction and resident intruder tests; Engin & Treit, 2008; File & Hyde, 1979; Millan, 2003; Rodgers, 1997), acute

responses (like freezing or startling to aversive stimuli; Engin & Treit, 2008; Millan, 2003), and exploration tests (Rodgers, 1997; Millan, 2003). Exploration paradigms take advantage of the conflict rodents experience between their aversion to light and open spaces as anxiety-provoking stimuli, and their simultaneous drive to explore novelty (Bourin et al., 2007; Crawley, 1981; Davis et al., 1997; Engin & Treit, 2008). In one common exploratory-based test of anxiety, the open field, an animal is placed into a well-lit open arena and its activity level and rearings are measured, with more rearing and more activity in the central area of the field suggested to indicate lower anxiety (Candland & Nagy, 1969; Homanics et al., 1999; Siemiatkowski et al., 2000). Another common exploration paradigm, the elevated plus-maze, is elevated off the ground and has two brightly lit open-sided arms and two dimly lit arms with high sides (Bourin et al, 2007; Engin & Treit, 2008; Hogg, 1996; Pellow et al., 1985). Rats forced to remain in the open arms show signs of stress, and the amount of time in the open arms can be decreased by anxiogenic drugs (Pellow et al., 1985; Pellow & File, 1986). Therefore, it has been suggested that a lower amount of time spent in the open arms indicates higher anxiety (Engin & Treit, 2008; Pellow et al., 1985).

An example of how the definitions of fear versus anxiety can be applied to behavioral paradigms assessing emotionality in animals involves another exploration test of anxiety, the light-dark box. The light-dark box is divided into a brightly lit light chamber, and a dimly lit, covered dark chamber (Bourin et al., 2007; Bourin & Hascoët, 2003; Crawley, 1981; Crawley & Goodwin, 1980). Subjects are placed in the light chamber and then allowed to move freely between the light and dark chambers through a connecting door (Bourin et al., 2007; Bourin & Hascoët, 2003; Crawley, 1981; Crawley & Goodwin, 1980). Common variables used to determine anxiety state are the latency to enter the dark chamber for the first time, the duration of

time spent in the light chamber, the number of transitions between the two chambers, and the duration of rearing (Bourin et al., 2007; Bourin & Hascoët, 2003). As mentioned previously, light is aversive for most rodents (File, 1980), and therefore, they spend less time in the brightly lit side of a light-dark box compared to the darker side, indicating that the light side is more aversive and more anxious animals are less likely to spend their time there, a finding that has been pharmacologically validated in male rats (Bourin & Hascoët, 2003; Crawley, 1981; Crawley & Goodwin, 1980). It might, at first, seem difficult to determine whether the light-dark box paradigm involves fear or anxiety, but utilizing the definitions given above and some further evidence makes it clear that this is an anxiety paradigm. In this apparatus, rodents are avoiding light which, while it is presumably often associated with actual threats, light itself is not harmful (Bourin et al., 2007). Furthermore, rodents exposed to bright light have increased plasma glucocorticoids, which can be blocked by a benzodiazepine that is anxiolytic in humans (File, 1980; File & Hyde, 1979), indicating that this response is likely due to anxiety and not fear. Davis and colleagues (1997) reviewed research showing that rodents exposed to bright light have long-term, exaggerated increases in acoustic startle, indicating that bright light is an anxiogenic stimulus to rodents. In addition, rats show greater startle in response to an acoustic stimulus when they are in a brightly lit environment compared to a dark one (Walker & Davis, 1997a). This effect is both sensitive to anxiolytic agents and is resistant to habituation (Walker & Davis, 1997a), meaning that light elicits long-term reactivity in rodents, which is indicative of anxiety. Finally, anxiolytic compounds increase the amount of time rodents spend in the brightly lit side of the light-dark box (Costall et al., 1989; Crawley & Goodwin, 1980), presumably demonstrating a decrease in anxiety behavior. Therefore, the light-dark box, and other related

paradigms such as the elevated plus-maze and open field, are tests specifically thought to target behavioral responses related to anxiety.

Neural sites involved in anxiety

Determining the neural correlates of anxiety is crucial for our understanding of this emotional state, and how to potentially alleviate it. Neuroanatomists and other researchers have been investigating possible neural sites relevant to anxiety for at least the last 100 years and have suggested a variety of areas likely to regulate this emotional state, both in humans and in other animals (Bremner, 2004; Cannon, 1927; Klüver & Bucy, 1939; Papez, 1937). Among all these brain regions, some of the most commonly studied and widely acknowledged regions involved in anxiety are the medial prefrontal cortex, amygdala, bed nucleus of the stria terminalis, hippocampus, and periaqueductal gray (Bremner, 2004; Cannon, 1927; Davidson et al., 2000a; Frewen et al., 2008; Papez, 1937; Ressler & Mayberg, 2007). To identify these possible neural sites that regulate anxiety in humans, more recently researchers have utilized some of the techniques discussed above, such as neuroimaging and studying patients with lesions (Bremner, 2004; Gorman et al., 2000; Davidson et al., 2000a; Davidson et al., 2002; Ressler & Mayberg, 2007). Neuroimaging studies have been a very useful tool to reveal differences in brain structure in patients that have anxiety disorders compared to control subjects (Bremner, 2004; Davidson et al., 2000b; Davidson et al., 2002; Ressler & Mayberg, 2007). While studies in humans do give us direct evidence of how brain activity changes in subjects whose anxiety states are changing, information about differences in brain activity in people with anxiety disorders, and reveal possible functions for brain regions that have been damaged or lesioned, there is a limit to the amount of information we can draw from these types of studies. To accomplish direct

experimental results about which brain regions are involved in anxiety and to gain experimentally driven data about how neural sites may be regulating anxiety, researchers must often turn to animal models both to help investigate neural correlates regulating anxiety and also to try to develop novel treatments or ways of understanding human anxiety disorders (Kent & Rauch, 2003; Von Bardeleben & Holsboer, 1988).

Many of the same brain regions purported to be involved in anxiety in humans have also been investigated in rodents, and in fact, a great deal of rodent work was conducted earlier than the human work and actually pointed researchers toward brain sites they should be studying in humans (Bremner, 2004; Kent & Rauch, 2003). In rodents, a variety of techniques have been utilized to identify brains regions that regulate anxiety. Chemical inactivation of or lesioning the amygdala, bed nucleus of the stria terminalis (BST), hippocampus, or periaqueductal gray (PAG), decrease fear and anxiety in many behavioral paradigms (Davis et al., 1997; Fendt et al., 1996; LeDoux et al., 1990; McEchron et al., 1998; Treit et al., 1993). When rodents are exposed to anxiety-generating paradigms, there is an increase in cells expressing Fos, the protein product of the immediate early gene *c-fos*, in the medial prefrontal cortex (mPFC), bed nucleus of the stria terminalis, amygdala, and periaqueductal gray, (Duncan et al 1996), indicating that these areas are involved in the animals' perception of or responses to these stimuli.

The central nucleus of the amygdala (CeA) is often associated with emotional responses, but its role specifically in anxiety is unclear. Lesions of the CeA block fear-potentiated startle and increase punished drinking (Campeau & Davis, 1995; Hitchcock & Davis, 1986; Iwata et al., 1986; LeDoux et al., 1988; Möller et al., 1997). There is no effect of CeA lesioning on behavior in elevated plus-maze behavior (Möller et al., 1997), though, but kindling of the posterior CeA does decrease open-arm exploration in the plus-maze (Adamec & Shallow, 2000). Lesions that

involve the basolateral and central nuclei of the amygdala disrupt passive avoidance of an electrified shock probe (Treit et al., 1993), as does infusion of the anxiolytic benzodiazepine midazolam into the CeA (Pesold & Treit, 1995). Furthermore, infusion of the anxiolytic agent, muscimol, into the CeA causes anxiolytic effects in the elevated plus-maze (Moreira et al., 2007), however contradictory results have also been reported (Zarrindast et al., 2008). These data indicate that GABA-mediated inhibition in the CeA might reduce anxiety-related behaviors, but further research is needed to clarify conflicting evidence.

The mPFC is involved in many aspects of fear learning and HPA activation in response to stressors (Akirov & Maroun, 2007), but it also plays a role in anxiety behavior (Duncan et al., 1996). Inactivation of the mPFC increases the amount of time rats spend in the open arms of an elevated plus maze (Shah et al., 2004) and depletion of dopamine in the mPFC using 6-OHDA decreases open arm time in this paradigm (Espejo 1997). In the BST, infusion of the AMPA receptor antagonist NBQX abolishes light-enhanced startle (Walker & Davis, 1997), and inhibiting GABA synthesis with L-allylglycine decreases time rats spend in the open arms of an elevated plus-maze (Sajdyk et al., 2008). Additionally, expression of the immediate-early gene *c-fos* is increased in the ventral BST in rats after exposure to the elevated plus-maze (Kabbaj & Akil, 2001; Smith & Lonstein, 2008), indicating involvement of this area in anxiety related to exploratory paradigms. The hippocampus has historically been studied mostly in relation to particular types of memory, but more recently there has been a great deal of work looking at the role the hippocampus plays in anxiety and other emotions (Bannerman et al., 2004; McHugh et al., 2004). For example, cytotoxic lesions of the hippocampus result in anxiolytic effects in the elevated plus-maze, the light-dark box and the social interaction tests of anxiety (Deacon et al., 2002; Kjelstrup et al., 2002; McHugh et al., 2004). Lesions of the PAG decrease freezing in

response to an anxiogenic predator stimulus and fear-potentiated startle (Behbehani, 1995; Fendt et al., 1996), while stimulation of this area elicits flight, defense, and anxiety responses (Behbehani, 1995, De Luca-Vinhas et al., 2006). Infusion of an NMDA receptor antagonist into the PAG decreases anxiety behavior in the plus-maze (Graeff et al., 1993). In terms of a larger neural anxiety network comprised of these sites, it is hypothesized that anxiety-generating stimuli activate the amygdala, which in turn projects to the PAG and activates it and then the PAG projects to many other brain regions and additionally to the spinal cord and stimulates anxiety, defense, and escape behaviors (Behbehani, 1995; Brandao et al., 2008; Gorman et al., 2000). Furthermore, although findings are not always consistent, it has been suggested that the medial prefrontal cortex acts to inhibit the amygdala and that the amygdala can influence output from the prefrontal cortex (Amstadter, 2008; Berkowitz et al., 2007). The hippocampus also projects to the amygdala, and this connection has been proposed to play a role in remembering the context of a fearful experience (LeDoux, 2000). These anatomical and pharmacological findings have led to a greater understanding of the neural correlates of animal anxiety and have provided valuable information concerning the organization of anxiety circuits in the rodent brain.

GABA and Anxiety

Many neurochemicals have been implicated to regulate anxiety in both humans and other animals (Davis, 1993; Hackler et al., 2007; Itoi, 2008; Landgraf & Wigger, 2002; Redmond et al., 1976; Stein & Stahl, 2000; Swanson et al., 2005; Treit et al., 1993; Wrenn & Crawley, 2001), but the inhibitory neurotransmitter gamma-aminobuytric acid (GABA) is particularly influential (Blanchard et al., 2003; Gray, 1983; Korff & Harvey, 2006; Miczek et al., 1995; Millan, 2003; Nemeroff, 2003 a,b; Roy-Byrnes, 2005). Of the three GABA receptor subtypes, there is much

more evidence for the $GABA_A$ receptor's involvement in the pathophysiology of anxiety than for the $GABA_B$ or $GABA_C$ receptors (Atack, 2003; Lydiard, 2003; Rudolph & Möhler, 2006; Schmidt, 2008; Smith, 2001; Whiting, 2006). Reduced expression or low sensitivity of central $GABA_A$ receptors is related to pathological anxiety in humans (Bremner et al., 2000a; Bremner et al., 2000b; Cowley et al., 1993; Cowley et al., 1995; Goddard et al., 2001; Malizia et al., 1998; Tiihonen et al., 1997) and high anxiety-related behaviors in laboratory animals (Concas et al., 1993; Rägo et al., 1991). Furthermore, in laboratory rodents, $GABA_A$ receptor agonists are anxiolytic in many behavioral paradigms including the elevated plus- and T-mazes, light-dark box, Vogel conflict test, and open-field (Bueno et al., 2005; Lippa et al., 2005; Nazar et al., 1997; Sienkiewicz-Jarosz et al., 2003; Yasumatsu et al., 1994).

$GABA_A$ receptors are widespread throughout the central nervous system but, not surprisingly, are concentrated in many regions implicated in anxiety, including the amygdala, BST, hippocampus, mPFC, and the PAG (Fénelon & Herbison, 1996; Liu & Glowa, 1999; Nelvokov, 2006; Roy-Byrne, 2005; Shah et al., 2004; Vermetten & Bremner, 2002a; Vermetten & Bermner, 2002b). Peripheral injections of $GABA_A$ receptor antagonists increase anxiety and Fos expression in many regions of the rat brain, including the amygdala, hippocampus, BST, and mpfc (Kurumaji et al., 2003; Salchner et al., 2006; Singewald et al., 2003), suggesting that anxiety behavior could be related to changes in GABA activity in these sites. In fact, anxiety is decreased when $GABA_A$ agonists and benzodiazepines are infused into the amygdala, hippocampus, mpfc, and PAG, while anxiety is increased after $GABA_A$ receptor inhibition in

these sites (Akirav et al., 2006; Bueno et al., 2005; Millan, 2003; Rezayat et al., 2005; Roy-Byrnes, 2005; Shah et al., 2004; Shah & Treit, 2004; Shibata et al., 1989).

The $GABA_A$ receptor is a pentameric structure composed of 5 distinct subunits situated around an ion channel that allows the passage of chloride ions into the cell, thereby hyperpolarizing and inhibiting that cell (Figure 1; Millan, 2003; Nutt, 2006; Roy-Byrnes, 2005). Therefore, $GABA_A$ receptor activation must decrease anxiety by inhibiting cells in brain regions that would normally contribute to an anxiety response when activated. There are at least 19 different subunits encoded by separate genes that can comprise the $GABA_A$ receptor (Nutt, 2006; Sieghart et al., 1999), with these receptors most commonly containing two α, two β, and one γ subunit, although δ, ε, and σ can substitute for the γ subunit (Da Settimo et al., 2007; Nutt, 2006). Each of these subunits and the different combinations of the subunits within the receptor affect the $GABA_A$ receptor's function and can differentially influence how GABA's binding to the receptor regulates anxiety (Burt, 2003; Korpi & Sinkkonen, 2006). For example, $GABA_A$ receptors containing α_1, α2, α3, or α5 subunits are highly sensitive to benzodiazepine-mediated effects, while those that are assembled with α4 or α6 subunits have a much lower affinity for benzodiazepines (Korpi & Sinkkonen, 2006; Nutt, 2006). Therefore, the receptor can be composed of many different subunit combinations, which can alter the receptor's function, and it is possible that differences in anxiety could be due to contributions from specific subunits or from different subunit combinations.

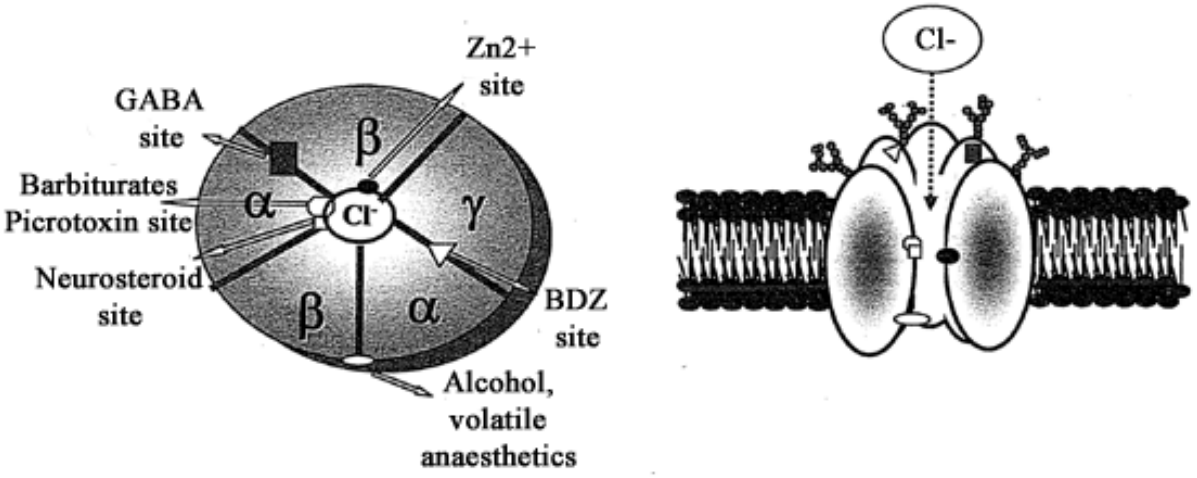

Figure 1. Schematic representations of the $GABA_A$ receptor. Reprinted from Da Settimo et al., 2007.

Sex differences and effects of hormones on anxiety

There are sex differences in human anxiety disorders, with women having a higher prevalence than men (Alexander et al., 2007; Altemus, 2006; Bekker et al., 2007; Wilson et al., 2004). This anxiety difference is greatest when women experience fluctuation in hormone levels, so it has been suggested that gonadal steroid hormones contribute to this emotional difference (Toufexis, 2007). While research on human anxiety has often involved study of both men and women, the rodent work on this topic largely neglects females. We do know that male rodents tend to display higher levels of anxiety behavior than female rodents (Adamec et al., 2006; Toufexis, 2007; Wilson et al., 2004), but that this is dependent on the hormonal state of the females during testing because the relatively small amount of research examining anxiety in female rodents reveals that a major influence is gonadal hormones (Toufexis, 2007; Wilson et al., 2004). Female rodents, like human women, experience changes in anxiety across different stages of the ovarian cycle. When female rodents are in proestrus, and their levels of estrogen

and progesterone are high, they exhibit less anxiety-like behavior than do diestrous females or male rodents (Toufexis, 2007; Marcondes et al., 2001). During diestrus, when progesterone levels are high and then fall, female rats exhibit increased anxiety that could be specifically due to the withdrawal effects of progesterone and possibly the resultant decrease in GABAergic activity in brain regions such as the PAG (Lovick, 2006). These estrous-cycle differences are abolished if estradiol is administered to diestrous females (Marcondes et al., 2001), and estradiol treatment decreases anxiety-related behavior in ovariectomized rats in multiple paradigms including the plus-maze (Walf & Frye, 2007), indicating that estrogen plays a role in mediating anxiety-related behavior. Additional evidence that both of these steroid hormones are involved in regulating anxiety-related behavior is that treatements with estradiol, progesterone, or estrogen followed by progesterone, all decrease anxiety in an elevated plus-maze and an open-field in ovariectomized female rats (Frye & Walf, 2004).

Progesterone has a rapid anxiolytic effect that is likely due to its rapid transformation to its metabolites in the brain (Galeeva et al, 2007; Lovick, 2006). Allopregnanolone, one of progesterone's reduced metabolites, can bind to the $GABA_A$ receptor and potentiate the inhibitory anxiolytic effects of GABA specifically in females depending on their stage in the estrous cycle (Pinna et al., 2000; Toufexis, 2007). This effect of progesterone on GABA-mediated modulation of anxiety is, at least in part, be regulated by the amygdala, as direct application of allopregnanolone to the amygdala of male and ovariectomized female rats reduces anxiety-related behavior in an elevated plus-maze, an open-field, and a modified Geller-Seifter punished responding test (Akwa et al., 1999; Frye & Walf, 2004). It has also been suggested that the periaqueductal gray (PAG) has altered excitability during different stages in the estrous

cycle, specifically during estrus and late diestrus, and that this phenomenon might be due to changes in $GABA_A$ receptor composition (Brack & Lovick, 2007).

Anxiety during pregnancy

Anxiety disorders during pregnancy in humans have received relatively little attention both compared to investigations of anxiety in non-pregnant subjects and to research on depression during this time period (Ross & McLean, 2006). However, there are some indications that there is a higher prevalence of some anxiety disorders, such as Obsessive-Compulsive Disorder and Generalized Anxiety Disorder, in pregnant women compared to the general population (Altshuler et al., 2000; Ross & McLean, 2006), and possibly an amelioration of symptoms of Panic Disorder during pregnancy (Altshuler et al., 2000). More extensive research on this topic is crucial, as anxiety disorders during pregnancy are indicated to cause a number of long-term problems in the infants of these women (Correia & Linhares, 2007; Glover, 1999; Misri & Kendrick, 2007; Ross & McLean, 2006; but see Littleton et al., 2007) and there are stressors and many unique aspects of this time period that could be important factors in treating anxiety disorders during the perinatal period (Glover, 1999; Littleton et al., 2007; Misri & Kendrick, 2007; Ross & McLean, 2006).

Female rodents also experience changes in emotional state throughout the pre- and peripartum periods (Tu et al., 2005). Similar to research on pregnant women, there have not been many studies performed to investigate anxiety during this time period in rodents and the research that has been accomplished is often contradictory. One study has found a decrease in anxiety-related behavior as pregnancy progresses as demonstrated through increased open arm time in the elevated plus maze on Days 14 and 19 of pregnancy, but not Days 7 or 21 in

comparison to ovariectomized virgin females (De Brito Faturi et al., 2006). However, there have also been somewhat contradictory findings that pregnant rats show decreased anxiety only on Day 18-19 of pregnancy, and instead show an increase in anxiety on both Days 15 and 21 in comparison to virgin rats (Neumann et al., 1998; De Brito Faturi et al., 2006) or that pregnant females do not show decreased anxiety on Day 18 compared to virgin females, but are more anxious at this later time point than during early pregnancy (Macbeth, 2008). Findings have also indicated no anxiety difference between early to mid-pregnant rats and virgin females (De Brito Faturi et al., 2006; Neumann et al., 1998), however contradictory results have also found decreased anxiety on Day 9 (Macbeth, 2008). In addition, when tested in a non-exploratory based anxiety paradigm, the shock probe defensive burying test, female rats show a decrease in anxiety behavior during the last week of pregnancy when compared to ovariectomized virgins (Picazo & Fernandez-Guasti 1993) or have been shown to display an increase in light enhanced acoustic startle compared to cycling and postpartum females and intact male rats (Toufexis, 2007). Levels of estrogen and progesterone increase throughout pregnancy and then fall just before parturition (Lonstein, 2007), and have been indicated for a role in regulating anxiety during pregnancy (De Brito Faturi et al., 2006; Neumann, 2003), but due to these contradictory findings on anxiety during pregnancy, any possible hormonal effects are difficult to consider. GABA content has been shown to decrease during late pregnancy (Altemus et al., 2004), but $GABA_A$ receptor binding has been demonstrated to increase along with neurosteroids during this time period (Ferreira et al., 1989; Follesa et al., 1998), possibly indicating a role for pregnancy-induced changes in GABA influencing anxiety-related behavior.

Anxiety in postpartum females

Female rodents are less anxious during the early postpartum period compared to virgin females (Fleming & Luebke, 1981; Lonstein, 2007; Toufexis, 2007; Toufexis et al., 1999), and lower anxiety is also seen in human mothers compared to pregnant and non-pregnant women (Altshuler et al., 2000; Breitkopf et al., 2006; Kendell et al., 1987; Kumar & Robson, 1984; Toufexis, 2007). Decreased anxiety in postpartum rats is exhibited in many behavioral paradigms, including the elevated plus-maze, open-field, punished drinking, shock-induced burying test, and acoustic startle test (Bridges et al., 1972; Ferreira et al., 1989; Fleming & Luebke, 1981; Hard & Hansen, 1985; Kellogg & Barrett, 1999; Lonstein, 2005; Picazo & Fernandez-Guasti, 1993; Smith & Lonstein, 2008; Toufexis et al., 1999; Toufexis, 2007). This postpartum decrease in anxiety-related behavior is not due to ovarian hormones, as ovariectomy following parturition does not prevent it (Hansen, 1990; Lonstein, 2005). However, infant contact is crucial for both rodent and human postpartum females to have decreased anxiety, as both human and rodent mothers allowed recent contact with their infants exhibit lower anxiety or anxiety-related behavior than postpartum humans and rodents without such recent contact (Heinrichs et al., 2001; Lonstein, 2005; Neumann, 2003). In rats, this infant-mediated reduction in anxiety requires direct physical contact, as placing the pups in a wire-mesh cage within the dam's homecage that allows only distal cues to reach her is not enough to reduce her anxiety (Lonstein, 2005). In contrast, reduced postpartum anxiety is not dependent on suckling by pups, as removal of the dams' teats during early lactation does not affect anxiety during the postpartum period (Lonstein, 2005). Neural sites of action that may be particularly relevant in infant-mediated modulation of anxiety have not been explored in detail. There is some indication that many of the same areas that are implicated in male anxiety are also involved in postpartum

anxiety, such as the bed nucleus of the stria terminalis, but sites that are not as commonly studied in males, such as the lateral habenula, may also play a role (Smith & Lonstein, 2008). The ventrocaudal PAG may be of particular importance, as lesions of this site in lactating rats further reduces their anxiety (Lonstein et al., 1998), indicating that this region normally stimulates anxiety-related behavior. Even though it is established that infant contact is necessary for both human women and female rodents to show reduced postpartum anxiety, and there is some evidence for neural sites of control, the mechanisms of how infant contact reduces anxiety are relatively unknown.

One neurochemical influence on this infant-mediated postpartum reduction in anxiety behavior in rats is increased GABAergic neurotransmission. Lactating dams allowed to interact with their pups have significantly higher cerebrospinal fluid (CSF) GABA levels compared to dams separated from their pups for just 6 hours (Qureshi et al., 1987). Separated dams' CSF GABA returns to control levels within 24 hours after reunion with pups (Qureshi et al., 1987), indicating that recent infant contact is necessary for increased GABA levels in lactating rats' central nervous systems. In addition, systemic injections of the $GABA_A$ inhibitor pentylenetetrazol decreases dams' punished drinking, which indicates an anxiogenic effect of this agent (Hansen, 1990), and lactating rats naturally exhibit more punished drinking, indicating lower anxiety, when compared to virgin female rats (Ferreira et al., 1989). Further evidence indicating GABA's involvement in decreased postpartum anxiety is that virgin female rats treated with benzodiazepines show similar freezing behavior in response to an anxiogenic noise burst as untreated lactating rats (Hansen et al., 1985). Additionally, peripheral administration of agents that inhibit the $GABA_A$ receptor (FG-7142 and pentylenetetrazol) potentiate mother rats' freezing in this paradigm (Hansen et al., 1985). Finally, when the $GABA_A$ antagonist,

bicuculline, is directly infused into the cPAGv of lactating rats and their anxiety level is tested in an elevated plus-maze, it increases to the level of diestrous virgin females (Miller, et al., 2010). Thus, higher GABA levels are part of the mechanism driving reduced anxiety during the postpartum period in rats with recent infant contact, however more work needs to be done to identify other brain regions (in addition to the cPAGv), where GABAergic activity may be particularly relevant for postpartum anxiety.

A recent study looking at differences between female rats that had never given birth (nulliparous) and those that were parturient and whose pups were ready to be weaned (primiparous) found that α2 subunit expression is significantly reduced in the medial amygdala in proestrous primiparous females compared to proestrous nulliparous controls, while α2 subunit expression in the PAG is higher in primiparous versus nulliparous proestrous females (Byrnes et al., 2007). This indicates that the α2 $GABA_A$ receptor subunit, and specifically its up- and downregulation after parturition, could somehow be involved in these brain sites to modulate postpartum anxiety. In a pseudopregnancy model, in which females are administered progesterone or its metabolites and then these neurosteroids are withdrawn to mimic hormone levels during pregnancy and parturition (Smith et al., 1998), $GABA_A$ current in the hippocampus decreases (Smith et al., 1998). However, studies that do not use naturally parturient and lactating rats might not be studying the phenomenon of interest because subjects in these studies do not go through the same neurochemical and biological changes as females that undergo parturition.

The ability of GABA to act on its receptor is vital for females to have decreased anxiety during the postpartum period. However, there are still many questions about its role that remain undiscovered. Though my dissertation work, I have further examined the relationship between neural GABA activity and postpartum anxiety. First, I demonstrate that the GABA-mediated

reduction in anxiety-related behavior during the postpartum period can be expanded to in another frequently utilized exploratory paradigm, the light-dark box; I further examined the relative importance of some of the different binding sites on the $GABA_A$ receptor contributing to this phenomenon (**Chapter 1**). Through the use of autoradiography, I then determined whether there are changes in neural $GABA_A$ receptor binding at three different ligand sites between the sexes and and across reproductive state in female rats, which could be related to observed differences between these groups in anxiety-related behavior (**Chapter 2**). Finally, I have utilized Western blots to determine whether neural $GABA_A$ receptor subunit composition differs among diestrous, pregnant, postpartum and sexually naïve male rats, which is also hypothesized to contribute to differences in binding affinity to the $GABA_A$ receptor as well as to the anxiety (**Chapter 3**). These findings contribute to our knowledge of the underlying neurobiological mechanisms of anxiety during the postpartum period in rats and will lead to future work to more fully define GABA's role in this suppression of anxiety during this unique phase of the reproductive cycle. Furthermore, the results of this research might even, at some point, influence research and treatment strategies for anxious postpartum women.

Chapter 1: Effects of ambient illumination level, litter contact, and $GABA_A$ receptor antagonism on behavior in the light-dark box

Introduction

Female rodents undergo complex behavioral changes during the peripartum period, including a decrease in anxiety-related behaviors (see Lonstein, 2007; Neumann, 2003) that may be a requirement for dams to attain heightened maternal responsiveness toward infants and aggression toward intruders (Fleming & Leubke, 1981; Hard and Hansen, 1985). Soon after parturition, postpartum females exhibit reduced anxiety when compared to pregnant or virgin females in many behavioral paradigms, including the punished drinking test, acoustic startle, T-maze, open field, elevated plus-maze, and defensive burying of an electrified probe (Bridges et al., 1972; Ferreira et al., 1989; Fleming & Luebke, 1981; Hard & Hansen, 1985; Kellogg & Barrett, 1999; Lonstein, 2005; Neumann, 2003; Picazo & Fernandez-Guasti, 1993; Smith & Lonstein, 2008; Toufexis et al., 1999). However, the light-dark box, a test very frequently used in male rodents over the past few decades (Blanchard and Canteras, 2008; Crawley, 1981; Crawley & Goodwin, 1980; File, 1980), has not been utilized to study reproductive state effects on anxiety nearly as often as other exploratory tests. Only three studies have compared the light-dark box behaviors of postpartum and virgin female rodents and they report contradictory results. Lactating female house mice were shown to be less anxious than virgin females in this apparatus (Maestripieri & D'Amato; 1991), but a more recent study suggests no significant effect of reproductive state on mouse light-dark box behavior (Gammie et al., 2008). Similarly, a recent study of female rats also found no significant effect of reproductive state on behavior in the light-dark box (Zuluaga et al., 2005).

It has also been demonstrated that infant contact is crucial for reduced anxiety during the postpartum period in rats (Lonstein, 2005; Neumann, 2003; Smith & Lonstein, 2008), as

separation from pups for as little as 4 hours increases postpartum females rats' anxiety to the level of diestrous virgins (Lonstein, 2005). In addition, postpartum dams that have been allowed continual access to their pups have significantly higher cerebrospinal fluid concentrations of GABA (Qureshi et al., 1987), implicating GABA in postpartum anxiety. In fact, inhibiting $GABA_A$ receptor activity increases postpartum females' freezing in response to an acoustic stimulus and also reduces their punished drinking (Hansen et al., 1985; Hansen, 1990).

Based on all the previous evidence indicating decreased anxiety in postpartum female rodents in many behavioral paradigms, but the discrepancy between the only three reports comparing light-dark box behavior across reproductive states, I have re-examined the usefulness of this paradigm for examining how anxiety-related behaviors are influenced by reproductive state. In Experiment Ia, I first examined whether different amounts of ambient light might affect anxiety differences between postpartum and diestrous rats, a methodological variability that could help explain previous discrepancies in reproductive state effects in the light-dark box (File & Hyde, 1979; Garcia et al., 2005; Morato & Castrechini, 1989; Valle, 1970). I demonstrated that postpartum rats are less anxious in a light-dark box than diestrous virgin females, confirming many previous reports of decreased postpartum anxiety (Fleming & Luebke, 1981; Hard & Hansen, 1985; Lonstein, 2005; Toufexis, 2007), but only when ambient illumination is high enough to be aversive to virgins. In Experiment Ib, I tested the prediction that recent sensory input from contact with pups is necessary for dams' reduced anxiety-related behavior in the light-dark box, as is the case in an elevated plus-maze (Figueira et al., 2008; Lonstein, 2005; Smith and Lonstein, 2008). In Experiment Ic, I investigated hypothesis that $GABA_A$ receptor activity is necessary for postpartum female rats to have lower anxiety behavior than diestrous virgins in the light-dark box. I did this by inhibiting the receptor at three different binding sites -

the GABA site (with (+)-bicuculline [(+)-bic]), the benzodiazepine site (with FG-7142), and the picrotoxin site (with pentylenetetrazol [PTZ]). As there are many peripheral $GABA_A$ receptors throughout the body, (Alam et al., 2006; Cairns et al., 1999; Carlton et al., 1999; Miñano et al., 1987; Ong and Kerr, 1990), I also determined whether any possible effects of these inhibiting agents were accomplished through central nervous system activity, by systemic injection of bicuculline methiodide [BM], a $GABA_A$ receptor antagonist that cannot cross the blood-brain barrier (Dalvi & Rodgers, 2001; Limmroth et al., 1996; Mareš et al., 2000; McDonald et al., 2008; Pong & Graham, 1972; Remler & Marcussen, 1985).

Experiment 1a: Effects of ambient illumination on light-dark box behavior of diestrous virgin and postpartum rats

Methods

Subjects

Subjects were female Long-Evans rats, born and raised in our colony and descended from rats purchased from Harlan Laboratories (Indianapolis, IN). After weaning at 21 days of age, subjects were group housed in clear polypropylene cages (48 x 28 x 16 cm) in groups of 2-3 female littermates, with wood shavings for bedding, a 12:12 light:dark cycle, and food and water available *ad lib*. After 70 days of age, subjects for the virgin groups were rehoused with 1-2 other non-sibling female virgins, while subjects for the postpartum groups were monitored daily with a vaginal impedance meter that measures changes in electrical resistance of the vaginal walls across the estrous cycle (Fine Science Tools, Foster City, CA). Females for the postpartum groups found to be in proestrus were mated overnight with sexually experienced males from our colony, then rehoused in groups of 2-3 pregnant females per cage the following day.

Approximately 4-5 days prior to the expected day of parturition, pregnant females were singly housed. Litters were culled to contain 4 males and 4 females within 48 hr after birth.

Light-dark box testing

Postpartum dams were tested on day 7 or 8 postpartum, with the day of parturition assigned as day 0. Virgin females were vaginally smeared daily to determine stage of the estrous cycle and were tested on a day of diestrus. Subjects were left undisturbed in the colony room for at least 3 hours prior to testing, then postpartum subjects were brought to a nearby testing room in their home cages and virgins were brought to the testing room in a clean cage so as not to disturb the other females housed with them. Subjects were then placed in the light chamber of a light-dark box. The light-dark box was made of white and black opaque Plexiglas with the dimensions of 20 x 30 x 30 cm for the light chamber and 30 x 30 x 30 cm for the dark chamber. The chambers were connected by a 10 x 10 cm door cut in the middle of the wall separating the two chambers.

Three different illumination levels were tested because previous studies utilizing the light-dark box in rodents differ in their illumination levels or do not report them (e.g., Bourin & Hascoet, 2003; Chaouloff et al., 1997; Hascoet & Bourin, 1998; Zuluaga et al., 2005). The high-illumination condition consisted of 1320 lux in the middle of the light chamber and 12 lux in the dark chamber (postpartum group $n = 9$; diestrous virgins $n = 8$). In the mid-illumination condition, the light chamber was 624 lux and the dark chamber was 3 lux ($n = 9$ postpartum females; $n = 8$ virgins). In the low-illumination condition, the light chamber was 15 lux and the dark chamber was 2 lux ($n = 6$ postpartum females; $n = 6$ virgins).

A mirror was placed above the light-dark box and the images in the mirror were videotaped with a Panasonic low-light sensitive video camera connected to a Panasonic VCR in an adjacent room. Females' behaviors were recorded for 10 minutes with a custom-made minicomputer data acquisition system either while being videotaped, or the videotapes were transcribed at a later time. For the high-illumination condition, observations were scored live by an observer seated ~2 m away from the apparatus, because the high intensity of the reflected light in the camera lens prevented videotaping. After testing, subjects were removed from the light-dark box and returned to the colony room. After each use, the box was cleaned with a 70% ethanol solution and allowed to dry completely before the next subject was tested.

Behavioral Variables

Behaviors in the light-dark box that were analyzed included the duration of time spent in the light chamber, number of full-body transitions between chambers, frequency of rears in the light chamber, frequency of stretches from the dark chamber into the light chamber (characterized by moving at least part of the head but not all four feet into the light chamber), the latency from the beginning of testing to enter the dark chamber, and the latency to re-enter the light chamber after the first bout of time spent in the dark chamber. These behaviors have all been previously measured as a reflection of anxiety in this apparatus (Costall et al 1989; Crawley et al., 1984; Crawley and Goodwin, 1980; Bourin and Hascoët 2003; De Angelis 1992; Hascoët and Bourin 1998; Lapin, 1999). An additional variable analyzed was the frequency of rears made in the light chamber standardized by the duration of time spent in that chamber.

Data Analysis

Data were analyzed with independent *t*-tests comparing postpartum and virgin rats tested under each light condition. Statistical significance was indicated by $p < 0.05$.

Results

Low- illumination

When subjects were tested under the low illumination condition, there were no significant differences between postpartum and diestrous females on any behavioral measure (Table 1), including the duration of time spent in the light chamber ($t(10) = 0.010$, $p > 0.99$; Table 1 and Figure 2).

Mid-illumination

Postpartum subjects tested in the mid-illumination condition spent significantly more time in the light chamber of the light-dark box than did diestrous virgins ($t(15) = 2.97$, $p = 0.0095$; Table 1 and Figure 2). Dams also transitioned between chambers significantly more frequently ($t(15) = 2.78$, $p < 0.014$) and reared more often ($t(15) = 2.93$, $p < 0.011$; Table 1) than did virgins. When the frequency of rears was standardized for the duration of time subjects spent in the light chamber, however, there was no significant difference between postpartum and virgin females ($t(11) = 1.37$, $p > 0.19$; Table 1).

There were no significant differences between dams and diestrous virgins in the frequency of stretches from the dark chamber to the light chamber ($t(15) = 0.57$, $p > 0.57$). There were also no significant differences between postpartum and virgin females in their latencies to make their first entry into the dark chamber ($t(15) = 0.73$, $p > 0.47$) or re-enter the

light chamber after having spent their first bout of time in the dark chamber ($t(15) = 1.02$, $p > 0.32$; Table 1).

High-illumination

Similar to what was found for the mid-illumination condition, postpartum females tested under high-illumination spent significantly more time in the light chamber compared to virgin females ($t(15) = 2.21$, $p = 0.043$; Table 1 and Figure 2). Dams also made significantly more chamber transitions ($t(15) = 3.21$, $p = 0.0058$) and reared more frequently ($t(12) = 2.67$, $p < 0.021$) than did virgins. When the frequency of rears was standardized for the duration of time spent in the light chamber, there was no difference between the groups ($t(9) = 0.83$, $p > 0.42$; Table 1).

There were no significant difference between postpartum and virgin females in the frequency of stretches from the dark chamber ($t(15) = 0.008$, $p > 0.99$). There was also no significant difference between groups in the latency to make the first entry into the dark chamber ($t(15) = 1.13$, $p > 0.27$; Table 1). However, after spending their first bout of time in the dark chamber, dams re-entered the light chamber significantly faster than did virgins ($t(15) = 2.35$, $p < 0.033$; Table 1).

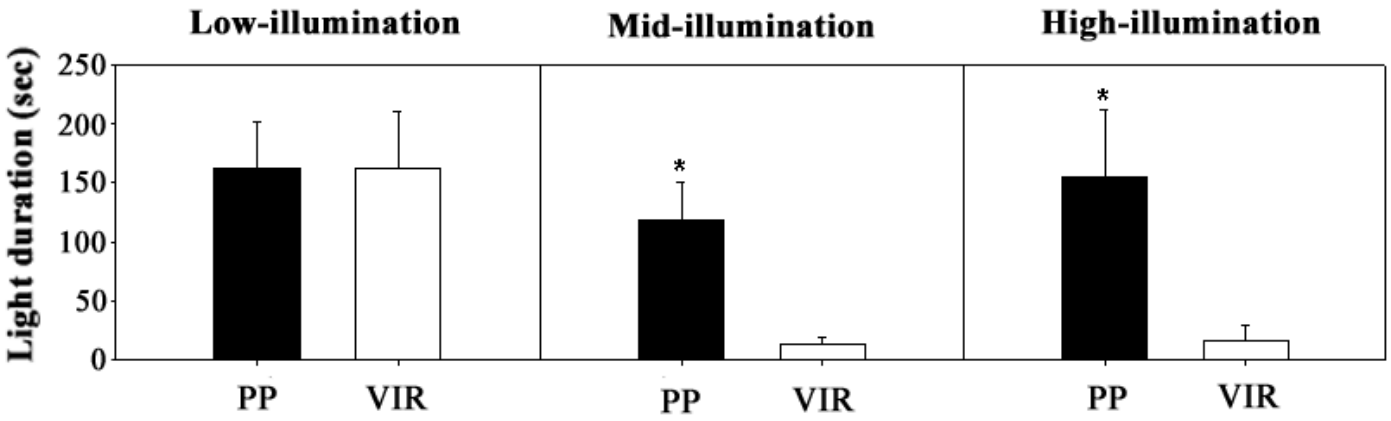

Figure 2. Duration of time (Mean +/- SEM) spent in the light chamber of a light-dark box by lactating (PP – black bars) and diestrous virgins (VIR – white bars) tested under three illumination levels. * indicates $p < .05$.

Table 1: Behavior of diestrous virgin and postpartum female rats tested in a light-dark box under three illumination levels.

	Low-Illumination				**Mid-Illumination**				**High-Illumination**			
	Virgin	Postpartum	*t*(10)	*p*	Virgin	Postpartum	*t*(15)	*P*	Virgin	Postpartum	*t*(15)	*P*
Time spent in Light Chamber	163 ± 47	162 ± 39	0.01	0.99	13 ± 6	118 ± 33	2.97	0.01	17 ± 13	155 ± 58	2.21	0.04
Number of Chamber Transitions	11 ± 2	16 ± 3	1.33	0.21	3 ± 1	9 ± 2	2.78	0.01	3 ± 0.9	9 ± 2	3.21	0.01
Frequency of Rears	24 ± 7	24 ± 7	0.00	1.00	2 ± 1	14 ± 4	2.93	0.01	2 ± 2	14 ± 3	2.67	0.02
Frequency of Rears/ Time spent in light chamber	15 ± 2	14 ± 2	0.59	0.57	9 ± 3	14 ± 2	1.37	0.20	10 ± 1	12 ± 2	0.83	0.43
Frequency of Stretches from Dark to Light	32 ± 5	39 ± 3	1.14	0.28	24 ± 3	27 ± 3	0.57	0.58	23 ± 6	22 ± 4	0.01	0.99
Latency to enter Dark chamber	24 ± 23	1 ± 0	0.99	0.35	2 ± 1	5 ± 3	0.73	0.48	1 ± 1	32 ± 25	1.13	0.28
Latency to re-enter Light Chamber	216 ± 95	137 ± 86	0.62	0.55	314 ± 107	178 ± 82	1.02	0.32	381 ± 107	98 ± 63	2.35	0.03

Note: Behavioral measures are means ± SEM, and given in seconds except for frequency variables. Statistical significance was indicated by $p < .05$.

Experiment 1b: Influence of litter contact on the light-dark box behavior of postpartum rats

Methods

Subjects

Subjects were postpartum rats from our colony that were housed as described in Experiment Ia.

Light-Dark Box Testing

Testing followed the same procedure as Experiment I, except that one group of lactating females (n = 15) had their pups removed and placed in an incubator set at 34^{o}C (ambient nest temperature) 4 hours before testing, which we have previously found to increase dams' anxiety-related behavior in an elevated plus-maze (Figueira et al., 2008; Lonstein, 2005; Smith & Lonstein, 2008). The other group of dams (n = 16) were left alone in their home cages and allowed continual contact with their pups until the time of testing. Litters were returned to separated dams after testing.

Data Analysis

Based on our previous data indicating that removal of the litter significantly increases dams' anxiety-related behaviors in an elevated plus-maze (Lonstein, 2005; Smith & Lonstein, 2008), the current data were analyzed using one-tailed t-tests, with statistical significance indicated by $p < 0.05$.

Results

Dams separated from their pups for four hours before testing spent significantly less time in the light chamber of a light-dark box compared to unseparated dams that were allowed continual access to their pups before testing ($t(35) = 1.77$, $p < 0.043$; Table 2 and Figure 3). Separated dams also exhibited significantly fewer chamber transitions than did dams allowed continual access to their pups before testing ($t(35) = 1.87$, $p < 0.036$; Table 2). These groups showed no significant differences on any other behavioral measures in the light-dark box, including the frequency of rears ($t(35) = 1.54$, $p > 0.066$; Table 2).

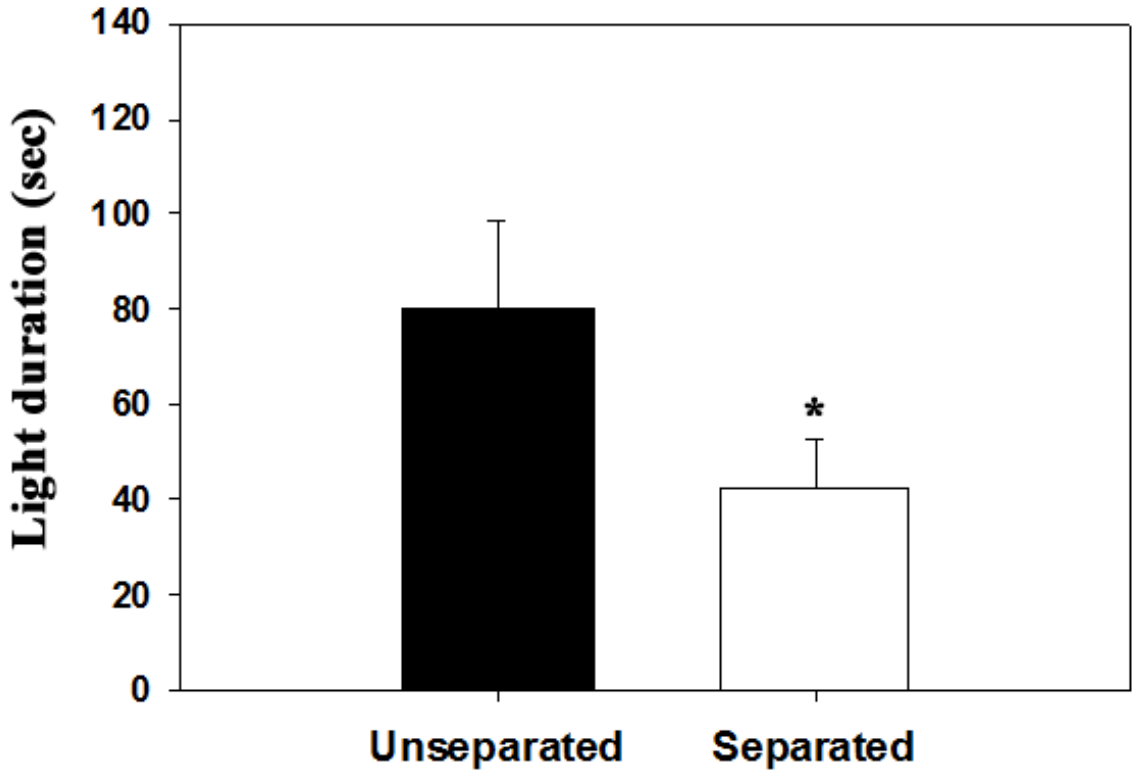

Figure 3. Duration of time (Mean ± SEM) spent in the light chamber of a light-dark box by postpartum rats that were either allowed access to their litters (Unseparated) or separated from their litters 4 hours before testing (Separated). * $p < 0.05$, one-tailed.

Table 2: Behavior of postpartum rats separated from pups or not four hr before testing in a light-dark box

	Unseparated	Separated	*t*(10)	*P*
Time spent in light chamber	80 ± 18	42 ± 11	1.77	0.04
Number of chamber transitions	10 ± 1	7 ± 1	1.87	0.04
Frequency of rears	12 ± 3	7 ± 2	1.54	0.07
Frequency of rears/ Time spent in light chamber	15 ± 1	14 ± 2	0.73	0.23
Frequency of stretches from dark to light	24 ± 2	24 ± 2	0.06	0.48
Latency to enter dark chamber	5 ± 4	1 ± 0	1.08	0.14
Latency to re-enter light chamber	142 ± 48	176 ± 53	0.48	0.32

Behavioral measures are means ± SEM, and given in seconds except for frequencies. Statistical significance was indicated by $p < .05$, utilizing one-tailed tests.

Experiment 1c: Effects of $GABA_A$ receptor inhibition on light-dark box behavior in postpartum and diestrous virgin rats

Methods

Subjects

Subjects were housed as in Experiment Ia, with the exception that virgins in this experiment were singly housed at least 3 days before testing. Our laboratory has previously found no effect of single versus same-sex sibling group housing on diestrous virgin females' behavior in an elevated plus-maze, and in both cases they display more anxiety-related behaviors than postpartum females (unpublished data).

Drugs

All drugs were purchased from Sigma (USA). (+)-Bicuculline [(+)-bic] (2 or 4 mg/kg) was prepared similarly to that described in McDonald et al. (2008). The drug was first dissolved in 45 μL acetic acid, 150 μL propylene glycol, and 200 μL NaOH (50%), the solution volume was then brought up to 0.8 mL with saline, pHed to 5, and then the solution volume was brought up to 1 mL, giving a reliably clear solution. FG-7142 (10 or 25 mg/kg) was dissolved in physiological saline with 1 drop of TWEEN-80 added per 2 mL solution, which was stirred and then sonicated for approximately 10 min before use. Pentylenetetrazol (PTZ) (10 or 20 mg/kg) and bicuculline methiodide (BM) (1 or 6 mg/kg) were dissolved in physiological saline. (+)-Bic and its vehicle were prepared fresh daily. FG-7142 was prepared fresh daily from stored vehicle. PTZ and BM were discarded if not used within a few days. The injection volume of all drug solutions was 1 ml/kg body weight. Control animals for each drug received the corresponding vehicle in which the drug was dissolved. To avoid the stress of handling and weighing on the day of testing, subjects were not weighed daily, but all were weighed within the 4 days before testing.

Light-Dark Box Testing

Light-dark box testing was conducted similarly to Experiment I above. Fifteen minutes before being brought to the testing room, subjects received an intraperitoneal injection of vehicle or drug in the colony room, and were then returned to their home cage until testing. Sample sizes were as follows: (+)-bic vehicle virgins (n = 16), postpartum (n = 13); (+)-bic 2 mg/kg virgins (n = 12), postpartum (n = 15); (+)-bic 4 mg/kg virgins (n = 12), lactating (n = 12); FG-7142 vehicle virgin (n = 12), lactating (n = 13); 10 mg/kg virgin (n = 16), lactating (n = 13); 25

mg/kg virgin (n = 18), lactating (n = 14); PTZ saline virgin (n = 12), lactating (n = 14); 10 mg/kg virgin (n = 11), lactating (n = 14); 20 mg/kg virgin (n = 12), lactating (n = 13); BM saline virgin (n = 10), lactating (n = 9); 1 mg/kg virgin (n = 13), lactating (n = 8); 6 mg/kg virgin (n = 12), lactating (n = 9).

Data Analysis

Data were analyzed with four 2 (reproductive state) x 3 (drug dose) ANOVAs, one for each of the drugs tested. Significant omnibus ANOVAs were followed by Fisher's LSD post-hoc tests comparing individual groups. Statistical significance was indicated by $p < 0.05$. One subject was eliminated from the 20 mg PTZ postpartum group after a Dixon's Q test revealed it as an outlier for the duration of time spent in the light chamber ($p < 0.01$).

Results

(+)-Bicuculline

Duration of Time Spent in the Light Chamber

There was a significant main effect of reproductive state on the duration of time spent in the light chamber, with dams spending significantly more time in the light chamber than virgins ($F(1,74) = 6.50$, $p < 0.013$; Table 3 and Figure 4). There was no main effect of (+)-bic ($F(2,74) = 2.25$, $p > 0.11$) and no interaction between reproductive state and (+)-bic ($F(2,74) = 0.85$, $p > 0.43$) on the duration of time spent in the light chamber (Table 3 and Figure 4).

Chamber Transitions

There was a main effect of reproductive state on the frequency of chamber transitions, with dams transitioning more than virgins ($F(1,74) = 17.81$, $p < 0.0001$; Table 3). There was also a main effect of (+)-bic ($F(2,74) = 3.50$, $p < 0.036$; Table 3), with post-hoc analysis revealing that vehicle-injected subjects transitioned more than subjects that received 2 mg of (+)-bic. There was no significant interaction between reproductive state and (+)-bic on the number of chamber transitions ($F(2,74) = 2.43$, $p > 0.094$; Table 3).

Rears

There was a main effect of reproductive state on the frequency of rears with dams rearing more than virgins ($F(1,74) = 7.48$, $p = 0.0078$; Table 3). There was no main effect of (+)-bic ($F(2,74) = 1.74$, $p > 0.18$) and no interaction between reproductive state and (+)-bic ($F(2,74) = 0.81$, $p > 0.44$) on the frequency of rears (Table 3). When frequency of rears was standardized for the duration of time subjects spent in the light chamber, there were no main effects of reproductive state ($F(1,47) = 0.60$, $p > 0.44$) or (+)-bic dose ($F(2,47) = 0.32$, $p > 0.72$), and no interaction between these factors ($F(2,47) = 0.38$, $p > 0.68$; Table 3).

Stretches

There were no main effects of reproductive state ($F(1,74) = 0.68$, $p > 0.41$) or (+)-bic ($F(2,74) = 1.79$, $p > 0.17$), and no interaction between these two factors ($F(2,74) = 1.25$, $p > 0.29$), on the frequency of stretches from the dark chamber (Table 3).

Latencies

There was a main effect of reproductive state on the latency to enter the dark chamber, with dams having a shorter latency than did virgins ($F(1,74) = 4.33$, $p < 0.41$; Table 3). There was no main effect of (+)-bic ($F(2,74) = 0.26$, $p > 0.76$), and no interaction between reproductive state and (+)-bic ($F(2,74) = 0.66$, $p > 0.51$), on this measure (Table 3). Dams did re-enter the white chamber significantly faster than virgins ($F(1,74) = 14.02$, $p = 0.0004$), but there was no main effect of (+)-bic ($F(2,74) = 1.87$, $p > 0.16$) and no interaction between reproductive state and (+)-bic on this measure ($F(2,74) = 2.23$, $p > 0.11$; Table 3).

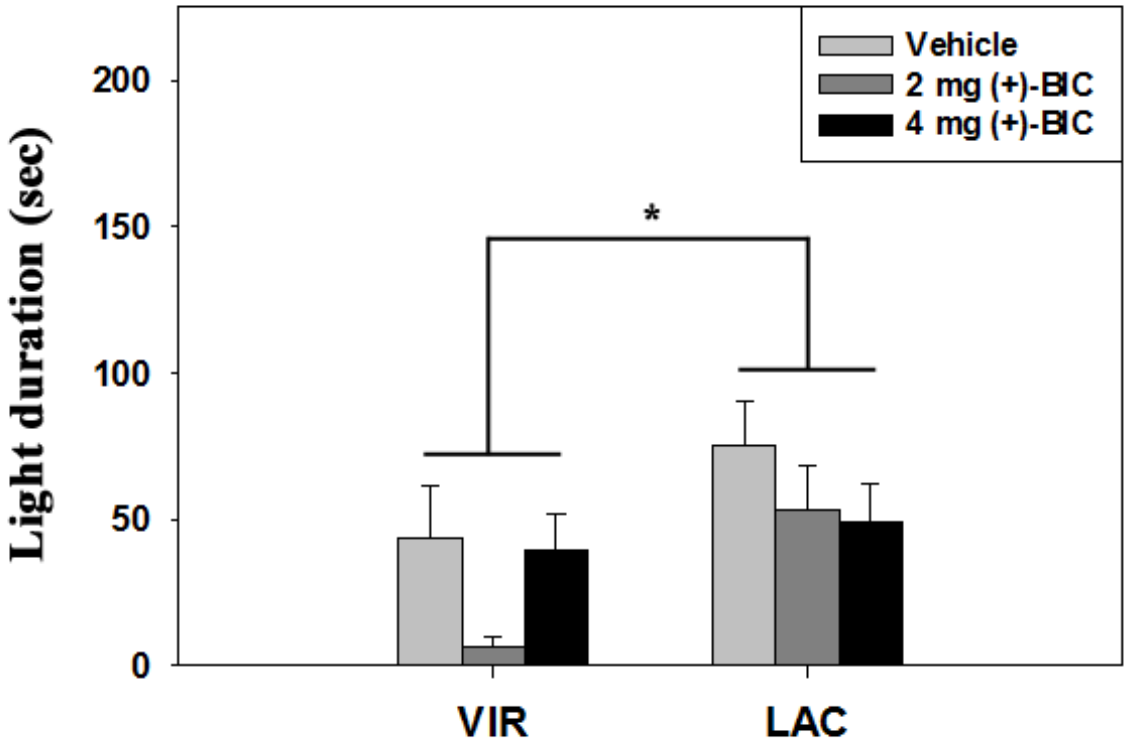

Figure 4. Duration of time (Mean ± SEM) spent in the light chamber by diestrous virgin (VIR) and lactating females (LAC – white bars) rats that received i.p. injections of vehicle, 2 mg (+)-bic, or 4 mg (+)-bic. * indicates a significant reproductive state difference.

Table 3. Behavior of diestrous virgin and postpartum female rats tested in a light-dark box after peripheral injection of Vehicle or (+)-Bic

	Virgin			**Postpartum**			**Significant Effects**
	Vehicle	(+)-Bic 2 mg	(+)-Bic 4 mg	Vehicle	(+)-Bic 2 mg	(+)-Bic 4 mg	
Time spent in light chamber	44 ± 18	7 ± 3	39 ± 13	76 ± 14	54 ± 14	49 ± 13	State
Number of chamber transitions	4 ± 1	2 ± 0	6 ± 2	11 ± 1	7 ± 1	7 ± 1	State, Dose
Frequency of rears	5 ± 2	1 ± 1	6 ± 2	10 ± 2	7 ± 2	7.5 ± 2	State
Frequency of rears/ Time spent in light chamber	11 ± 1	13 ± 4	13 ± 2	14 ± 0	13 ± 1	14 ± 2	----------
Frequency of stretches from dark to light	25 ± 3	21 ± 2	26 ± 4	31 ± 2	24 ± 3	23 ± 3	----------
Latency to enter dark chamber	6 ± 3	3 ± 2	7 ± 4	0 ± 0	2 ± 2	2 ± 1	State
Latency to re-enter the light chamber	418 ± 60	466 ± 69	245 ± 81	86 ± 45	228 ± 71	196 ± 71	State

Behavioral measures are means ± SEM given in seconds, except for frequency variables.

FG-7142

Duration of Time Spent in the Light Chamber

There were no main effects of reproductive state ($F(1,80) = 1.24$, $p > 0.26$) or FG-7142 dose ($F(2,80) = 0.66$, $p > 0.51$), and no interaction between these factors ($F(2,80) = 0.27$, $p > 0.76$), on the duration of time females spent in the light chamber (Table 4). However, there was high variability in both dams and virgins receiving FG-7142, which probably prevented a significant effect of reproductive state. In fact, vehicle-injected dams did spend almost three-fold more time in the light chamber than did vehicle-injected diestrous virgins (Figure 5).

Chamber Transitions

The frequency of chamber transitions was significantly higher in dams than in virgins ($F(1,80) = 17.28$, $p < 0.0001$). There was no significant main effect of FG-7142 ($F(2,80) = 1.77$, $p > 0.17$) and no significant interaction between reproductive state and FG-7142 on transitions ($F(2,80) = 1.46$, $p > 0.23$; Table 4).

Rears

There was a significant main effect of reproductive state on the frequency of rears with dams rearing more than virgins ($F(1,80) = 13.51$, $p = 0.0004$), but no main effect of FG-7142 dose ($F(2,80) = 0.63$, $p > 0.53$) and no interaction between the factors on rearing ($F(2,80) = 2.33$, $p > 0.10$; Table 4). When the frequency of rears was standardized for the amount of time subjects spent in the light chamber, there were no significant main effects of reproductive state ($F(1,58) = 0.068$, $p > 0.79$) or FG-7142 ($F(2,58) = 0.088$, $p > 0.91$), and no interaction between these factors ($F(2,58) = 0.49$, $p > 0.61$; Table 4).

Stretches

There was no main effect of reproductive state on the frequency of stretches from the dark chamber to the light chamber ($F(1,79) = 2.28$, $p > 0.13$; Table 4). When collapsed across reproductive state, there was a main effect of FG-7142 on the frequency of stretches, such that the 10 mg and 25 mg doses both significantly reduced the number of stretches when compared to vehicle ($F(2,79) = 6.71$, $p = 0.002$; Table 4). There was no significant interaction between reproductive state and FG-7142 on the frequency of stretches from the dark chamber ($F(2,79) = 0.23$, $p > 0.79$; Table 4).

Latencies

There were no main effects of reproductive state ($F(1,80) = 1.11$, $p > 0.29$) or FG-7142 ($F(2,80) = 1.32$, $p > 0.27$) on the latency to enter the dark chamber, and no interaction between these factors ($F(2,80) = 0.65$, $p > 0.52$; Table 4). However, there was a main effect of reproductive state on the latency to re-enter the light chamber after spending time in the dark chamber, with virgins taking longer to re-enter the light side compared to dams ($F(1,79) = 11.34$, $p = 0.0012$; Table 4). There was no significant main effect of FG-7142 ($F(2,79) = 1.15$, $p > 0.32$), and no significant interaction between reproductive state and FG-7142 ($F(2,79) = 2.50$, $p > 0.088$; Table 4) on the latency to re-enter the light chamber.

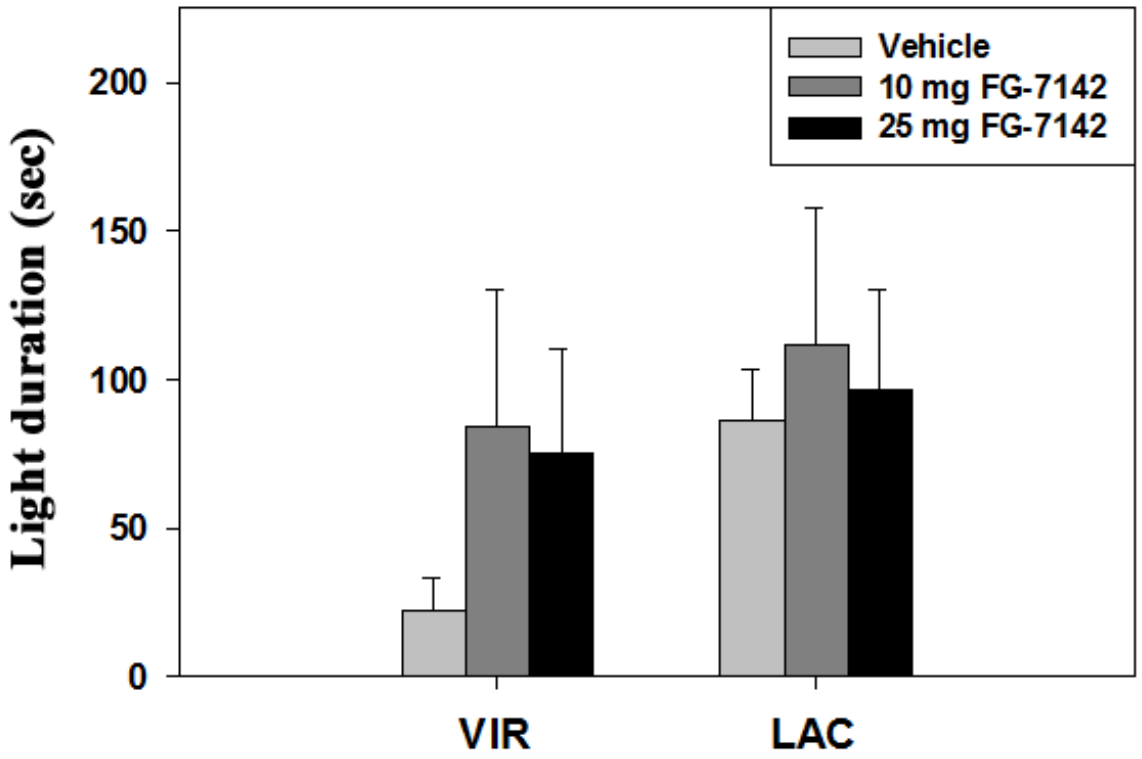

Figure 5. Duration of time (Mean ± SEM) spent in the light chamber by diestrous virgin (VIR) and lactating females (LAC) rats that received i.p. injections of vehicle, 10 mg FG-7142, or 25 mg FG-7142.

Table 4. Behavior of diestrous virgin and postpartum female rats in a light-dark box after peripheral injection of Vehicle or FG-7142

	Virgin			**Postpartum**			**Significant Effects**
	Vehicle	FG-7142 10 mg	FG-7142 25 mg	Vehicle	FG-7142 10 mg	FG-7142 25 mg	
Time spent in light chamber	22 ± 11	85 ± 46	80 ± 37	86 ± 16	102 ± 49	97 ± 34	----------
Number of chamber transitions	4 ± 1	3 ± 1	4 ± 1	10 ± 1	6 ± 1	8 ± 2	State
Frequency of rears	2 ± 1	5 ± 2	3 ± 1	12 ± 3	6 ± 2	8 ± 2	State
Frequency of rears/ Time spent in light chamber	9 ± 3	11 ± 3	10 ± 2	12 ± 1	10 ± 2	9 ± 1	----------
Frequency of stretches from dark to light	16 ± 4	14 ± 3	20 ± 4	32 ± 2	20 ± 4	27 ± 4	Dose
Latency to enter dark chamber	4 ± 2	50 ± 37	11 ± 4	1 ± 0	11 ± 7	7 ± 6	----------
Latency to re-enter the light chamber	431 ± 67	309 ± 75	338 ± 69	103 ± 39	135 ± 64	301 ± 65	State

Note: Behavioral measures are means ± SEM, and given in seconds except for frequency variables.

Pentylenetetrazol

Duration of Time Spent in the Light Chamber

Dams spent significantly more time in the light chamber compared to virgins ($F(1,67) = 5.07$, $p < 0.028$; Table 5 and Figure 6). There was a marginally significant main effect of PTZ, with the 20 mg dose tending to decrease the duration of time spent in the white chamber compared to 10 mg of PTZ or saline ($F(2,67) = 3.05$, $p = 0.054$; Table 5 and Figure 6). There was a significant interaction between reproductive state and dose of PTZ, with dams that received the 20 mg dose of PTZ spending less time in the light chamber compared to dams that received the 10 mg dose or saline, whereas there was little effect of PTZ on virgins ($F(2,67) = 3.88$, $p < 0.026$; Table 5 and Figure 6).

Chamber Transitions

Postpartum females made significantly more chamber transitions than did virgins ($F(1,68) = 16.13$, $p = 0.0002$), and there was also a main effect of PTZ with the 20 mg dose significantly reducing chamber transitions ($F(2,68) = 7.73$, $p = 0.0009$; Table 5). Additionally, there was an interaction between reproductive state and PTZ, with 20 mg of PTZ reducing chamber transitions compared to 10 mg of PTZ or saline in dams only ($F(2,68) = 7.10$, $p = 0.0016$; Table 5).

Rears

The frequency of rears was significantly higher in dams compared to virgins ($F(1,68) = 9.02$, $p = 0.0037$; Table 5). There was also a main effect of PTZ, with the 20 mg dose

significantly reducing the frequency of rears compared to either saline or 10 mg PTZ ($F(2,68) = 4.74$, $p < 0.012$; Table 5). Additionally, there was an interaction between reproductive state and dose, with dams, but not virgins, showing reduced rearing after injection of the 20 mg dose of PTZ but not after the 10 mg dose or saline ($F(2,68) = 5.44$, $p = 0.0064$; Table 5). When the frequency of rears was standardized for the duration of time subjects spent in the light chamber, there was no main effect of reproductive state ($F(1,41) = 2.08$, $p > 0.15$) but there was a significant main effect of dose ($F(2,41) = 4.71$, $p < 0.015$) with 20 mg of PTZ significantly reducing the standardized frequency of rears compared to the 10 mg dose or saline ($F(2,41) = 4.71$, $p < 0.015$). There was no significant interaction between reproductive state and PTZ on the standardized frequency of rearing ($F(2,41) = 0.31$, $p > 0.73$; Table 5).

Stretches

There were main effects of reproductive state and PTZ on the frequency of stretches from the dark chamber to the light. Dams stretched more often than did virgins ($F(1,68) = 5.02$, $p < 0.029$), and all subjects receiving 20 mg of PTZ displayed fewer stretches than those receiving 10 mg of PTZ or saline ($F(2,68) = 15.87$, $p < 0.0001$). There was no significant interaction between reproductive state and PTZ on stretching ($F(2,68) = 2.05$, $p > 0.13$; Table 5).

Latencies

There was a significant main effect of reproductive state on the latency to enter the dark chamber with dams faster to enter the dark chamber than virgins ($F(1,68) = 4.08$, $p < 0.048$), but there was no main effect of PTZ ($F(2,68) = 0.83$, $p > 0.44$) and no interaction between these factors on this measure ($F(2,68) = 0.18$, $p > 0.83$; Table 5). There was also a main effect of

reproductive state on the latency to re-enter the light chamber after the first bout of time spent in the dark chamber, with dams re-entering the light chamber faster than did virgins ($F(1.68) = 15.24$, $p = 0.0002$). PTZ also had an effect with subjects given 20 mg of PTZ taking significantly longer to re-enter the light chamber than did those receiving either 10 mg PTZ or saline ($F(2,68) = 8.75$, $p = 0.0004$). Lastly, there was a significant interaction between reproductive state and PTZ on the latency to re-enter the light chamber, with 20 mg of PTZ increasing the latency specifically in dams ($F(2,68) = 5.27$, $p = 0.0074$; Table 5).

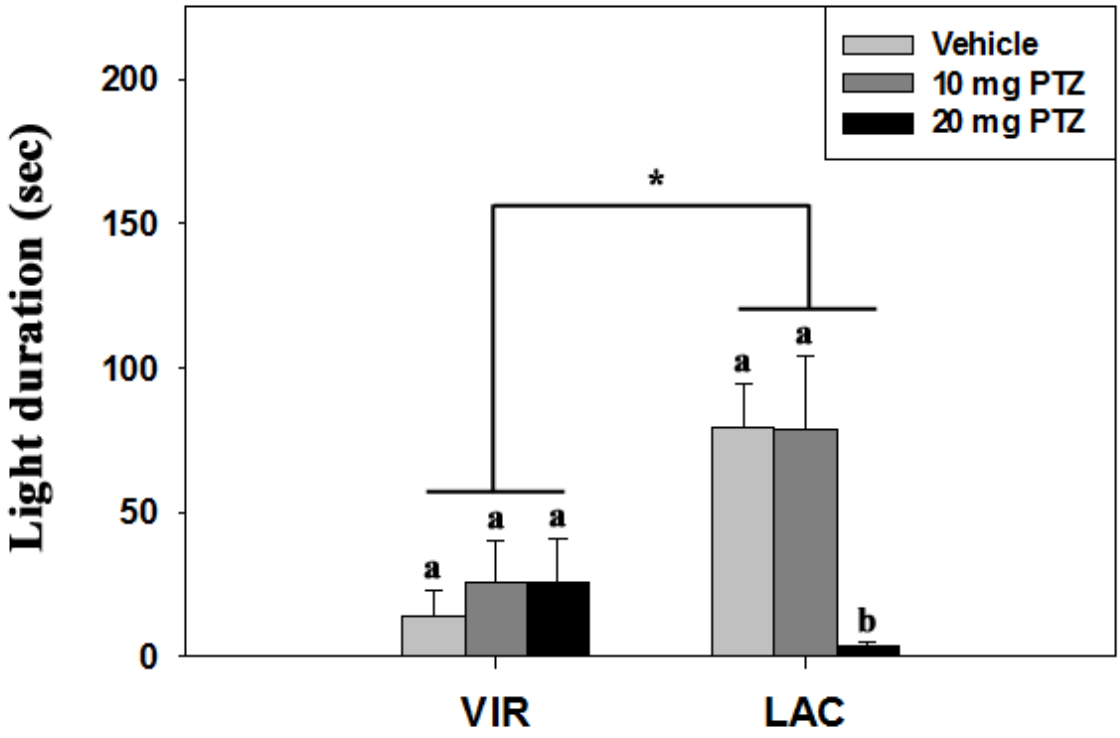

Figure 6. Duration of time (Mean ± SEM) spent in the light chamber by diestrous virgin (VIR) and lactating females (LAC) rats that received i.p. injections of vehicle, 10 mg PTZ, or 20 mg PTZ. *significant main effect of reproductive state, $p < .05$. Different letters above bars indicate significant difference within each reproductive state.

Table 5: Behavior of diestrous virgin and postpartum female rats in a light-dark box after peripheral injection of Vehicle or PTZ

	Virgin			**Postpartum**			**Significant Effects**
	Vehicle	10 mg	20 mg	Vehicle	10 mg	20 mg	
Time spent in light chamber	15 ± 9	29 ± 15	26 ± 15	80 ± 15	79 ± 26	4 ± 2	State, State x Dose
Number of chamber transitions	3 ± 1	3 ± 1	3 ± 1	12 ± 2	9 ± 1	2 ± 1	State, Dose, State x Dose
Frequency of rears	2 ± 1	3 ± 1	2 ± 2	12 ± 2	8 ± 2	0 ± 0	State, Dose, State x Dose
Frequency of rears/ Time spent in light chamber	55 ± 22	45 ± 15	16 ± 14	87 ± 7	59 ± 12	27 ± 25	Dose
Frequency of stretches from dark to light	23 ± 4	16 ± 5	9 ± 2	29 ± 3	28 ± 2	9 ± 3	State, Dose
Latency to enter dark chamber	2 ± 1	4 ± 1	3 ± 2	1 ± 0	2 ± 1	1 ± 1	State
Latency to re-enter the light chamber	389 ± 76	429 ± 86	459 ± 66	114 ± 46	94 ± 22	492 ± 65	State, Dose, State x Dose

Note: Behavioral measures are means ± SEM, and given in seconds except for frequency variables.

Bicuculline Methiodide

Duration of Time Spent in the Light Chamber

There was a significant main effect of reproductive state on the duration of time spent in the light chamber, with dams spending more time there than virgins ($F(1,60) = 14.23$; $p = 0.0004$; Table 6 and Figure 7). There was no significant main effect of BM ($F(2,60) = 1.13$, $p > 0.33$), or interaction between reproductive state and BM, on time spent in the light chamber ($F(2,60) = 0.82$, $p > 0.44$; Table 6 and Figure 7).

Chamber Transitions

There was a significant main effect of reproductive state on the number of chamber transitions, with dams transitioning more than virgins ($F(1,60) = 19.94$, $p < 0.0001$). There was no main effect of BM on this measure ($F(2,60) = 2.36$, $p > 0.10$), nor an interaction between reproductive state and dose ($F(2,60) = 1.14$, $p > 0.32$; Table 6).

Rears

Postpartum females reared more than virgins ($F(1, 60) = 16.80$, $p = 0.0001$; Table 6). There was no significant main effect of BM on the frequency of rears ($F(2,60) = 1.82$, $p > 0.17$) and no interaction between reproductive state and BM ($F(2,60) = 1.48$, $p > 0.23$; Table 6). When this variable was standardized for the amount of time subjects spent in the light chamber there were no significant main effects of state ($F(1,50) = 0.72$, $p > 0.40$) or BM ($F(2,50) = 1.20$, $p > 0.14$), and no significant interaction between these factors ($F(2,50) = 1.11$, $p > 0.33$; Table 6).

Stretches

There were no significant main effects of reproductive state ($F(1,60) = 0.30$, $p > 0.58$) or BM ($F(2,60) = 0.45$, $p > 0.64$) on the frequency of stretches from the dark chamber, and no significant interaction between these factors ($F(2,60) = 0.046$, $p > 0.96$; Table 6).

Latencies

There were no main effects of reproductive state ($F(1,60) = 2.15$, $p > 0.15$) or BM ($F(2,60) = 0.69$, $p > 0.50$) on the latency to enter the dark chamber. There was a significant interaction between reproductive state and BM on the latency to enter the dark chamber ($F(2,60) = 3.39$, $p < 0.041$); Table 6), but post-hoc analyses revealed no significant difference between any two groups. There was a main effect of reproductive state on the latency to re-enter the light chamber after the first bout of time spent in the dark chamber, with lactating subjects re-entering the light chamber significantly faster than did virgins ($F(1,60) = 4.15$, $p = 0.046$). There was no main effect of BM on the latency to re-enter the light chamber ($F(2,60) = 0.14$, $p > 0.87$) and no interaction between state and BM ($F(2,60) = 0.56$, $p > 0.57$; Table 6) on this latency.

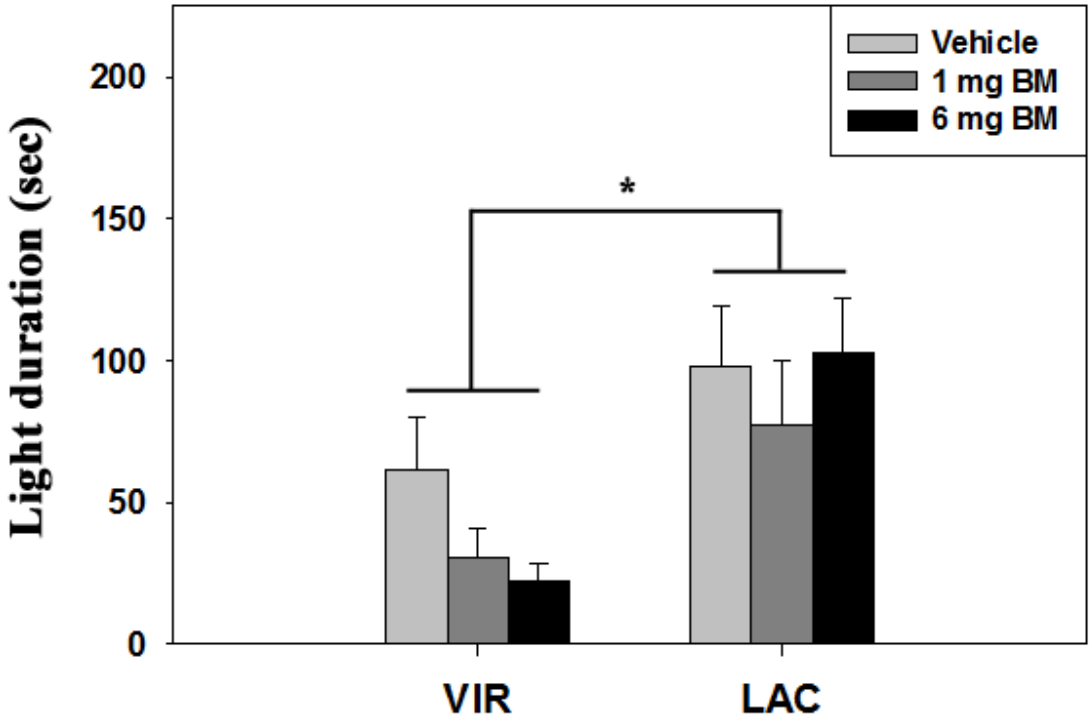

Figure 7. Duration of time (Mean ± SEM) spent in the light chamber by diestrous virgin (VIR) and lactating females (LAC) rats that received i.p. injections of vehicle, 1 mg BM, or 6 mg BM. *significant main effect of reproductive state at $p < .05$.

Table 6: Behavior of diestrous virgin and postpartum female rats in a light-dark box after peripheral injection of Vehicle or BM

	Virgin			**Postpartum**			**Significant Effects**
	Vehicle	1 mg	6 mg	Vehicle	1 mg	6 mg	
Time spent in light chamber	62 ± 19	31 ± 10	23 ± 6	98 ± 22	78 ± 22	103 ± 20	State
Number of chamber transitions	8 ± 2	3 ± 1	5 ± 1	12 ± 3	8 ± 2	10 ± 2	State
Frequency of rears	7 ± 2	2 ± 1	5 ± 1	11 ± 3	9 ± 3	15 ± 4	State
Frequency of rears/ Time spent in light chamber	10 ± 2	12 ± 5	15 ± 2	13 ± 2	12 ± 2	16 ± 1	----------
Frequency of stretches from dark to light	28 ± 4	31 ± 5	29 ± 4	28 ± 2	27 ± 4	28 ± 4	----------
Latency to enter dark chamber	18 ± 9	2 ± 2	0 ± 0	0 ± 0	6 ± 3	3 ± 2	State x Dose
Latency to re-enter the light chamber	175 ± 72	267 ± 77	268 ± 82	146 ± 55	118 ± 56	112 ± 54	State

Note: Behavioral measures are means ± SEM, and given in seconds except for frequency variables.

Discussion

The present experiment demonstrated that: 1) postpartum rats display fewer anxiety-related behaviors in a light-dark box than do diestrous virgins when tested under mid- or high-illumination, but not when tested under low ambient illumination, 2) postpartum dams allowed access to their pups until testing display fewer anxiety-related behaviors in a light-dark box compared to dams separated from their pups for the 4 hours before testing, and 3) $GABA_A$ receptor antagonism with (+)-bic and FG-7142 produced some minor anxiogenic effects in both dams and virgins, but blockade of the picrotoxin site with PTZ was strongly anxiogenic, and often selectively in dams.

Comparison with previous reports – Methodological considerations

Our experiments demonstrate that early postpartum rats display lower levels of anxiety-related behavior in the light-dark box - including more time spent in the light chamber, more transitions between chambers, and more frequent rearing - when compared to diestrous virgins. These results are consistent with previous work using many other behavioral paradigms to test anxiety-related behavior across reproductive states (for review see Lonstein, 2007). However, there have been only three previous studies examining how postpartum state affects behavior in a light-dark box and the results are inconsistent. Similar to our results, Maestripieri and D'Amato (1991) found that postpartum mice spend more time in the light chamber of a light-dark box than do virgin females (estrous females excluded). However, Gammie and colleagues (2008) recently found no significant difference between postpartum and virgin female mice (estrous stage undetermined) in their time spent in the light chamber, latency to enter the light chamber, or the number of chamber transitions in the light-dark box. Zuluaga and colleagues (2005) also found

no significant differences between postpartum and diestrous virgin rats in the duration of light chamber time, latency to first enter the light chamber, or the number of chamber transitions made in the light-dark box.

There have been considerable inter-laboratory differences in the procedures used for light-dark box testing (Bourin & Hascoët, 2003; Hascoët et al., 2001; Hascoët & Bourin, 1998; Takao & Miyakawa, 2006) and such differences may help explain contradictions among the now four studies of postpartum rodent behavior in this paradigm. For example, different strains of mice (Homanics et al., 1999; Takahashi et al., 2008; Van Gaalen & Steckler, 2000; Crawley et al., 1997; Crawley & Davis, 1982; Van Gaalen & Steckler, 2000; although see Bourin & Hascoët, 2003) and rats (Ramos et al., 1997; Rex et al., 1999; Shepard and Myers, 2008; Valle, 1970) differ in their anxiety. Making a comparisons between postpartum and virgin outbred Swiss mice (Mastripieri and D'Amato, 1991) and C57BL/6J mice (Gammie et al., 2008), or between Wistar (Zuluaga et al., 2005) and Long-Evans (present study) rats, is difficult. C57BL/6 mice, used by Gammie and colleagues (2008), are less anxious than some other mouse strains (Homanics et al., 1999; Takahashi et al., 2008; Van Gaalen & Steckler, 2000) including the Swiss mice studied by Maestripieri and D'Amato (1991) (Crawley et al., 1997; Crawley & Davis, 1982; Van Gaalen & Steckler, 2000; although see Bourin & Hascoët, 2003). In fact, the virgin group in the Gammie et al. (2008) study spent somewhat more time in the light chamber of the light-dark box than the respective group in the Maestripieri and D'Amato (1991) study. It could be that this increased time spent in the light chamber by CJ5BL/6 virgins decreased the possibility of finding a significant reproductive state effect. However, there might be other factors involved as the virgins' light chamber duration was not only not significantly different

from postpartum females, it actually appeared to be higher than that of postpartum females (Gammie et al., 2008).

Another methodological factor differing between the studies in mice is that the dams tested by Gammie and colleagues' (2008) had previously been tested in the light-dark box as virgins. Repeated testing in exploratory paradigms has been seen to increase anxiety-related behavior in subsequent tests (Bertoglio & Carobrez, 2000; Dos Reis & Canto-de-Souza, 2008; Griebel et al., 1993; Nosek et al., 2008; although see Hogg, 1996) and Gammie and colleagues (2008) did state that repeated testing could have affected their subjects' anxiety-related behavior. A further consideration for Gammie and colleagues' (2008) study is that it appears that virgins' stage the estrous cycle was unknown. Anxiety behaviors change drastically across the estrous cycle (Lovick, 2006; Marcondes et al., 2001; Mora et al., 1996; Toufexis, 2007; Zuluaga, 2005), so even if diestrous virgins were more anxious than dams, virgins in less anxious stages could have increased the virgin group's overall average light chamber duration such that it was higher than lactating females.

Lastly, the chamber in which subjects are placed to start the light-dark box test may be particularly important, as starting chamber affects the degree of aversiveness to the light chamber in male rats (Chaouloff et al., 1997). Both Zuluaga and colleagues (2005) and Gammie et al., (2008) started their tests with subjects placed in the dark chamber and found no effect of postpartum state, while we and Maestripieri and D'Amato (1991) placed subjects in the light chamber at the start of testing. In fact, I have data (Miller and Lonstein, 2006) indicating that female rats spend much less time in the light chamber when they are originally placed in the dark chamber (43±26 sec in light) than if they are placed in the light chamber at the beginning of testing (165±42 sec in light).

Ambient Light Levels Influence Light-Dark Box Behavior

Experiment 1a has demonstrated that lactating rats are less anxious than diestrous virgin rats in the light-dark box when tested at mid- or high-illumination conditions, but not under low-illumination. This was due to changes in the virgin females' behavior in response to the amount of light, as they spent less time in the light chamber under the two higher ambient light intensities than at the lowest illumination condition. The intensity of the ambient light has previously been seen to affect anxiety-related behaviors of male rodents tested in the light-dark box and other paradigms (Bertoglio & Carobrez, 2002; Costall et al., 1989; Garcia et al., 2005; Griebel et al., 1993; Hascoët et al., 2001; Valle, 1970; although see Becker & Grecksch, 1996; Pellow et al., 1985). The effects of ambient light levels on anxiety behavior have also been examined in female rats, but only in virgins (Mora et al., 1996). My data are the first to examine this question in rats during the postpartum period. It was found that postpartum rats showed a high duration of time spent in the light chamber even under very intense illumination, which attests to the robustness of the postpartum reduction in anxiety. Because differences in ambient light intensity had such a strong effect on the light-dark box behavior of our diestrous virgins, it seems possible that procedural differences among light-dark box studies in amount of ambient light could contribute to whether or not postpartum state affects anxiety-related behavior in this paradigm. Unfortunately, it is difficult if not impossible to determine exactly what light levels were used in previous studies (most of which do not report this in lux).

Infant Contact Influences Light-Dark Box Behavior

Postpartum female rats allowed access to their pups prior to testing displayed less anxiety-related behavior in the light-dark box when compared to dams that had their pups removed 4 hours before testing. This finding supports previous reports showing or suggesting that recent infant contact is required for the postpartum reduction in anxiety when tested with an elevated plus-maze (Lonstein, 2005; Lonstein & Smith, 2008; Neumann, 2003). While the underlying neurobiological causes of how infant touch affects mothers' anxiety behaviors is not entirely defined, a number of neurochemicals likely affect this phenomenon. Indeed, central GABA, norepinephrine, serotonin, prolactin, oxytocin, and corticotrophin releasing hormone are all transiently modified when mothers touch their infants (Lonstein, 2007; Miller and Lonstein, 2008; Neumann, 2003). It is worth mentioning that the effects of litter removal on dams' anxiety-related behavior in the light-dark box were not as strong as what our laboratory previously observed in animals tested in an elevated plus-maze. Some studies find that behavior in these two paradigms are strongly correlated (Henderson et al., 2004; Ramos, 2008), but other work suggests that the light-dark box is not as sensitive as the elevated plus-maze to some potentially anxiety-modulating manipulations including injection of well-established anxiogenic and anxiolytic agents, and that behavior in the two paradigms could reflect different undefined aspects of an animal's emotional state (Biala & Kruk; 2007; Hascoët & Bourin, 1998; McCool & Chappell, 2007; Ramos, 2008; Zuluaga et al., 2005).

$GABA_A$ Receptor Influences on Light-Dark Box Behavior

When dams are allowed to interact with pups, not only do they show low anxiety behavior, they also have significantly higher cerebrospinal fluid (CSF) concentrations of GABA

than when they have not been allowed recent offspring contact (Qureshi et al., 1987). Work from the Hansen laboratory suggested that this elevated GABA neurotransmission is involved in the postpartum reduction in anxiety, as they found that $GABA_A$ receptor antagonism with pentylenetetrazol or FG-7142 increases postpartum females' freezing in response to an acoustic stimulus and pentylenetetrazol reduces their punished drinking to levels found in virgins (Hansen et al., 1985; Hansen, 1990). This chapter has expanded upon these findings by demonstrating that $GABA_A$ receptor inhibition in diestrous virgin and lactating female rats can, in some cases, also increase anxiety-related behaviors in a light-dark box. It is particularly notable that the three $GABA_A$ antagonists did not produce identical effects on females' behavior. Whereas GABA site blockade with (+)-bicuculline and benzodiazepine site inverse agonism with FG-7142 did not significantly affect either postpartum or virgin rats' time spent in the light chamber (a primary measure of anxiety-related behavior in the light-dark box; Bourin & Hascoët, 2003), agonism of the picrotoxin site with PTZ, which inhibits the receptor, significantly affected this measure in dams but not virgins. This is probably not due to the choice of (+)-bicuculline and FG-7142 doses, which were in the range previously observed to affect anxiety-related behaviors in rodents (Atack et al., 2005; Evans & Lowry, 2007; Hansen et al., 1985; Nicolas & Prinssen, 2006; Pellow & File, 1986; Rodgers et al., 1995; Varty et al., 2002; Akirav et al., 2006; Nutt, 2006; Rodgers & Dalvi, 1997; Roy-Byrnes, 2005; Shah et al., 2004; Zarrindast et al., 2001; Zagrodzka et al., 2000), particularly because these agents did have some anxiogenic effects, but they just were not as anxiogenic in a light-dark box as PTZ. Furthermore, $GABA_A$ receptor inhibitors, including (+)-bic and FG-7142, can inconsistently affect anxiety behavior (Rodgers and Dalvi, 1997; Sanders & Shekhar, 1995; Zarrindast et al., 2001, 2008; Hart et al., 1998;

Hascoët and Bourin, 1998; Millan, 2003; Risbrough and Geyer, 2005). In fact, our lab has previously shown that the same 4 mg/kg dose of (+)-bicuculline increases anxiety-related behavior in dams tested in an elevated plus-maze (Miller et al., 2010). There may simply be test-dependent effects of $GABA_A$ receptor ligands on anxiety-related behavior, as suggested previously for male rodents (Nazar et al., 1997).

In contrast to (+)-bicuculline and FG-7142, PTZ was strongly anxiogenic on most variables measured in the light-dark box, and affected only postpartum females for many of them. PTZ binds to the picrotoxin site to inhibit GABAergic activity (Dibas & Dillon, 2000; Huang et al., 2001), and has anxiogenic effects in male rodents in many behavioral paradigms (Cruz et al., 1994; Cole et al., 1995; De Angelis, 1992; Giusti et al., 1991; Jones et al 2002; Pellow et al., 1985; Rodgers et al., 1995). It also increases punished drinking (Hansen, 1990) and acoustic startling in postpartum rats (Hansen et al., 1985). PTZ affects the $GABA_A$ receptor by binding to the picrotoxin site that lies within the open chloride channel (Bali and Akabas, 2007; Huang et al., 2001) and when the picrotoxin site is bound and GABA leaves its binding site, the channel is slower to reopen when GABA binds again (Bali and Akabas, 2007). Thus by affecting the picrotoxin site, PTZ may have even stronger anxiogenic potential than antagonists that bind to GABA's own site, because PTZ both blocks the chloride channel and slows its reopening, while other ligands must bind competitively to sites outside the channel pore. The fact that the higher dose of PTZ greatly affected some anxiety-related behaviors only in postpartum females implies that normal suppression of the picrotoxin site on the $GABA_A$ receptor deserves particular attention in mediating reduced anxiety during the postpartum period.

In addition to the influences of central $GABA_A$ receptors on behavior, there are also peripheral $GABA_A$ receptors on tissues of the joints and limbs (Cairns et al., 1999; Carlton et al., 1999), and throughout the digestive system (Miñano et al., 1987; Ong and Kerr, 1990) interfere with nociception (Cairnes et al., 1999; Carlton et al., 1999), protect against gastric ulcers (Miñano et al., 1987), and may be involved in intestinal motility (Ong and Kerr, 1990). To determine whether these findings that $GABA_A$ receptor antagonism has anxiogenic effects in postpartum rats were due to central receptor inhibition or had some peripheral receptor contributions as well, the $GABA_A$ receptor antagonist, bicuculline methiodide [BM], which does not readily cross the blood-brain-barrier (Dalvi and Rodgers, 2001; Limmroth et al., 1996; Mareš et al., 2000; Pong and Graham, 1972; Remler and Marcussen, 1985) was used. BM had no readily interpretable effects (there was an interaction between reproductive state and BM on the latency to enter the dark chamber, but post-hoc analysis revealed no significant difference between any two groups), suggesting little role for the GABA binding site on peripheral $GABA_A$ receptors in the light-dark box behavior of either postpartum or virgin female rats.

In conclusion, these results have shown that postpartum rats are less anxious in a light-dark box than diestrous virgins, and that this postpartum reduction in anxiety is dependent on ambient illumination, recent infant contact and high transmission at the $GABA_A$ receptor. $GABA_A$ receptor site of action affects postpartum anxiety behaviors differentially, and mediation at the picrotoxin site (as opposed to GABA's own site or the benzodiazepine site) may be particularly relevant for regulating postpartum anxiety. Because postpartum females were more responsive to inhibition through the picrotoxin site than diestrous virgins, there must be a

natural reproductive state difference driving this differential responsivity. Two possible mechanisms could be either a natural reproductive state difference in density of neural picrotoxin sites or a difference in affinity mediated by differences in $GABA_A$ receptor subunit concentrations in brain regions regulating anxiety. The first possible mechanism was examined in Chapter 2 through use of autoradiographic assay.

Chapter 2: Autoradiographic analysis of GABA, benzodiazepine, and picrotoxin binding sites in postpartum, pregnant, diestrous virgin, and sexually naïve male rats

Introduction

Chapter 1 indicates that antagonism of three different binding sites of the $GABA_A$ receptor has different effects on anxiety-related behavior particularly in postpartum rats, including strong anxiogenic effects due to inhibition of the receptor through the picrotoxin site. In addition, there have been some other studies indicating that activity at the $GABA_A$ receptor is necessary for postpartum females to show lower anxiety behavior (Hansen et al., 1985; Hansen, 1990; Miller et al., 2010). These data suggest that GABA neurotransmission at the $GABA_A$ receptor is necessary for postpartum rats to have reduced anxiety compared to diestrous virgins, and that the picrotoxin binding site is of particular importance. In contrast, diestrous virgins, who exhibit much higher anxiety-related behavior than postpartum rats in many paradigms, did not show as broad an increase in anxiety-related behavior due to $GABA_A$ receptor antagonism in a light-dark box in Chapter 1. These findings indicate that there is likely a natural state difference in the $GABA_A$ receptor system in the brains of postpartum females compared to diestrous virgins that allows dams to have lower anxiety behavior and also to be more responsive to the effects of picrotoxin site ligands.

One difference in the GABAergic system likely includes an increase in GABAergic neurotransmission in the postpartum brain. Qureshi et al., (1987) demonstrated that central GABA levels are high when dams are with pups, and this could be partly responsible for their decreased anxiety because pup contact is also critical for dams to have reduced anxiety. However, increased GABA release might only occur in some brain regions that regulate anxiety

while other regions may be different in postpartum brains in another way, or increased GABA release might only be part of the mechanisms driving lower postpartum anxiety. Differences in $GABA_A$ receptor density could also contribute to this phenomenon, as, for example, termination of pseudopregnancy increases both anxiety behavior and [^{3}H]flunitrazepam binding in the hippocampus (Bitran & Smith, 2005). There have been two previous studies investigating $GABA_A$ receptor binding affinity across reproductive states, though they leave many questions unanswered. Ferreira et al. (1989) investigated [^{3}H]muscimol ([^{3}H]MUSC), [^{3}H]flunitrazepam ([^{3}H]FNP), and [^{35}S]t-butylbicyclophosphorothionate ([^{35}S]TBPS) binding in homogenized brains of Day 5-8 postpartum rats and compared them to cycling virgins. They found no significant differences in $GABA_A$ receptor binding with these three radioligands, however they used large tissue punches and do not report binding of all three radioligands in all brain regions. [^{3}H]MUSC was examined in the cortex, hippocampus, and cerebellum, while [^{3}H]FNP binding was investigated in the cortex, hippocampus, amygdala, hypothalamus, and olfactory bulbs, and [^{35}S]TBPS binding was only examined in the olfactory bulbs (Ferreira et al., 1989). Majewska and colleagues (1989) also examined [^{3}H]MUSC binding in the brains of postpartum (Day 2) and cycling virgin rats, as well as females sacrificed at different time points during pregnancy. They used homogenates of the entire forebrain to look at both binding affinity and total binding density and found a decrease in $GABA_A$ receptor GABA binding site density in postpartum females in comparison to late pregnant females, but this was accompanied by an increase in [^{3}H]MUSC binding affinity in these Day 2 postpartum females, indicating that they have fewer

GABA binding sites, but a higher affinity to those GABA sites. They did not find a binding density difference between Day 2 postpartum rats and cycling virgins (Majewska et al., 1989). Unfortunately, these findings can only be applied to the rat forebrain as a whole or to a few large areas of the brain, without any more detailed site specificity. While these studies give some indication that overall forebrain levels of $GABA_A$ receptor binding might not differ across reproductive states, or may not contribute to the postpartum decrease in anxiety, there is a great need for a re-examination of binding to these $GABA_A$ receptor sites. These previous two studies did not compare Day 7 postpartum rats with diestrous virgin rats, which is the paradigm necessary to see reduced postpartum anxiety behavior. In addition, these studies did not give a very site-specific examination of binding in brain regions associated with emotional behaviors, which is particularly vital to understanding GABAergic mechanisms in the female brain that regulate anxiety behavior.

Although these two past studies do not show binding site density differences between differentially anxious postpartum and virgin females, alterations of $GABA_A$ receptor binding has been shown to affect anxiety behavior in rodents. In rats, systemic injection of allopregnanolone each morning for 48 hours alters the ability of benzodiazepines to bind to the $GABA_A$ receptor and also increases anxiety behavior in an elevated plus-maze in both males and females, although note that acute exposures of progesterone and its metabolites are anxiolytic (Gulinello & Smith, 2003). The anxiogenic effects of short-term neurosteroid administration are suggested to be due to the changes in binding affinity to the $GABA_A$ receptor, a possibility that Majewska and colleagues (1989) also mention. Furthermore, as mentioned above, termination of pseudopregnancy in female rats results in increased anxiety behavior and also alters binding to

the $GABA_A$ receptor benzodiazepine site (Bitran & Smith, 2005). These findings indicate that a more site-specific re-examination of $GABA_A$ receptor binding in the neural anxiety network in female rats across different reproductive states would be valuable, particularly because density of the apparently very important picrotoxin site (Chapter 1) has not been examined across reproductive states in any brain region aside from the olfactory bulb (Ferreira et al., 1989).

To examine whether $GABA_A$ receptor binding differs in the brains of postpartum rats versus diestrous virgins, I have performed autoradiography using [^{3}H]MUSC to assess binding to the GABA site, [^{3}H]FNP to determine benzodiazepine site binding, and [^{35}S]TBPS to investigate picrotxin site binding. Although my results from Chapter 1 indicate that the most relevant $GABA_A$ receptor binding site for postpartum light-dark box anxiety behavior is the picrotoxin site, and that the GABA and benzodiazepine sites are not as important, these binding sites have been demonstrated to be involved in postpartum anxiety behavior in other paradigms (Hansen et al., 1985; Miller et al., 2010). Therefore, I also examined binding density of these sites for their relevance to postpartum anxiety in these other anxiety paradigms. In addition to postpartum and diestrous females, I also examined binding of these three radioligands in the brains of mid-pregnant (Day 10 pregnancy) rats, a time point when females have undergone some of the hormonal changes of pregnancy, but do not have significantly different anxiety behavior than virgins (De Brito Faturi et al., 2006; Neumann et al., 1998; although see Macbeth, 2008). As mid-pregnant females do not differ in their anxiety behavior from virgins, they should not differ from virgins in their $GABA_A$ receptor binding density if binding density is in fact related to anxiety behavior, so looking at this reproductive state not only provides a more complete

understanding of the GABAergic system across multiple reproductive states, but also offers a control for whether early pregnancy hormonal changes can influence $GABA_A$ receptor binding without influencing anxiety behavior. A group of sexually naïve male rats was also included to investigate possible sex differences, given that their anxiety behavior differs from that of postpartum females, but is similar to diestrous females' anxiety (Fleming & Luebke, 1981; Toufexis, 2007; Toufexis et al., 1999). The mPFC, BST, CeA, Hipp, rPAG, and cPAG were all examined, as these brain regions regulate anxiety behavior through GABAergic neurotransmission. From my chapter 1 results, I hypothesized that postpartum females were likely to have either less or more [^{35}S]TBPS binding in one or more of the investigated brain regions in comparison to diestrous virgins. Chapter 1 results indicate that there could be differential binding in the brains of postpartum females, but this difference could be either an increase or a decrease. As postpartum females were more sensitive to the effects of PTZ, a picrotoxin site agonist, it is possible that they have a higher number of picrotoxin binding sites to account for that increased sensitivity, but that they naturally have a much lower amount of endogenous picrotoxin site agonists, such as purines and pyrimidines like hypoxanthine and inosine (Olsen, 1981; Olsen et al., 1980; Olsen & Leeb-Lundberg, 1980;), than diestrous females, which would account for their normally lower anxiety. However, it is also possible that postpartum females actually have fewer picrotoxin binding sites because then postpartum females could be more sensitive to small amounts of picrotoxin agonist because even a small concentration might be able to bind to and affect all of their picrotoxin sites, whereas a small addition of picrotoxin agonists to diestrous virgins might not have an effect if they already have more endogenous picrotoxin site agonists. Unfortunately the content of picrotoxin site agonists has not been compared between postpartum and diestrous virgin rats, so without first looking at

picrotoxin binding density and then, perhaps in the future, looking at endogenous picrotoxin agonists, it is difficult to determine the likelihood of these hypotheses. In addition, it is hypothesized that postpartum females will also have either lower or higher [^{35}S]TBPS binding in numerous brains sites than sexually naïve males, as postpartum females also have reduced anxiety levels compared to virgin males (Fleming & Luebke, 1981; Toufexis, 2007; Toufexis et al., 1999). Lastly, I expect a sex difference between virgin females and males in [^{3}H]MUSC binding, such that virgin males will have more [^{3}H]MUSC binding in some of these brain regions, as found by Kokka and colleagues (1992) in the cortex of intact or gonadectomized male and female rats. Kokka et al. (1992) did not find a sex difference in [^{35}S]TBPS or [^{3}H]FNP binding, though, and I predict that I will also not find a sex difference in binding of these two radioligands.

Methods

Subjects

Subjects were adult female and male Long-Evans rats born and raised in our laboratory, descended from rats purchased from Harlan Laboratory (Indianapolis, IN). After weaning at day 21 of age, subjects were housed in groups of 3-4 same-sex littermates in clear polypropylene cages (48 × 28 × 16) with wood shavings for bedding, food and water available *ad libitum*, and a 12:12 h light/dark cycle. Beginning at day 70 of age, females' estrous cycles were monitored daily with a vaginal impedance meter (Fine Science Tools, Foster City, CA) for the pregnant and lactating group subjects or were vaginally smeared to determine stage of the estrous cycle for diestrous virgin subjects. At this time, subjects for the virgin group were singly housed for at

least 3 days prior to sacrifice, and then sacrificed on a day of diestrus. Subjects for the male group were also singly housed at least 3 days prior to sacrifice. Subjects for the pregnant and lactating groups were mated with sexually experienced Long-Evans male rats from our colony overnight on a day of proestrus. After mating, these females were housed with other mated females, 2-3 per cage. Subjects for the pregnant group were separated from their cagemates on day 7 of gestation, and then sacrificed on pregnancy day 10 (±1 day). Lactating subjects were individually housed approximately 5 days before parturition, and their litters were culled to contain 8 pups (4 females and 4 males) within 48 hours after parturition. Lactating females were sacrificed on day 7 (±1 day) postpartum. Separation of virgins and males, and mating of pregnant and lactating subjects was timed such that some subjects from all groups were sacrificed on the same days.

Tissue collection and sectioning

On a day of diestrus for virgin subjects (n = 8), day 10 (±1 day) of pregnancy (n = 8), day 7 (±1 day) postpartum (n = 8), or the same day as other groups for males, subjects were rendered unconscious though exposure to CO_2 gas for < 1 minute, then rapidly decapitated using a guillotine. Their brains were removed immediately and placed on dry ice, then stored at -80^{o}C until sectioning. Brains were coronally sectioned on a cryostat at 15 μm in a one-in-six series of sections at -20^{o}C and thaw-mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). After sectioning and mounting, slides were stored at -80^{o}C until further processing.

Autoradiography

Slides were removed from the -80°C freezer and allowed to thaw at room temperature for 1 hour. For the [^{3}H]MUSC and [^{3}H]FNP assays, slide-mounted sections were first fixed in 1% paraformaldehyde for 2 minutes and then preincubated twice in 50 mM Tris-HCl buffer (pH 7.4) at 4°C for 15 minutes each. Sections were then incubated in a solution containing 50 mM Tris-HCl buffer and either 10 nM [^{3}H] muscimol (20-40 Ci/mmol; PerkinElmer, Waltham, MA) or 2 nM [^{3}H] flunitrazepam (70-87 Ci/mmol; Perkin-Elmer, Waltham, MA) for 1 hour at 4°C. For the [^{35}S]TBPS assay, sections were preincubated at room temperature twice for 15 minutes each in 1 mM EDTA and 120 mM NaCl in 50 mM Tris-HCl buffer (pH 7.4), then washed for 15 minutes at room temperature in 120 mM NaCl in 50 mM Tris-HCl (pH 7.4). Slides were then incubated in 0.5 nM [^{35}S]TBPS (>60 Ci/mmol; Perkin-Elmer, Waltham, MA) for 1 hour at room temperature. Nonspecific binding was determined by incubating adjacent sections in the presence of 100 μM GABA (Sigma-Aldrich, St. Louis, MO), 10 μM flunitrazepam (Sigma-Aldrich, St. Louis, MO), or 100 μM picrotoxin (Sigma-Aldrich, St. Louis, MO), for [^{3}H] muscimol, [^{3}H] flunitrazepam, or [^{35}S] TBPS, respectively. After incubation, sections were washed twice in 4°C 50 mM Tris-HCl buffer for 30 seconds each and then dipped for a few seconds in 4°C distilled H_2O for the [^{3}H] muscimol and [^{3}H] flunitrazepam slides or washed twice in room temperature 50 mM Tris-HCl buffer for 5 minutes each and then dipped for a few seconds in room temperature distilled H_2O for the [^{35}S] TBPS slides. Slides were laid out to dry overnight at 4°C for the [^{3}H] muscimol and [^{3}H] flunitrazepam procedures, or at room

temperature for the [^{35}S] TBPS slides. The following day, slides were moved to autoradiography cassettes (Fisher Scientific, Pittsburgh, PA) each containing a set of tritium microscale standards (3-110 nCi/mg and 0.1-16 nCi/mg; Perkin-Elmer, Waltham, MA) for the [^{3}H] muscimol and [^{3}H] flunitrazepam slides and a set of ^{14}C microscale standards (0.00 - 35.0 nCi/mg; American Radiolabeled Chemicals, St. Louis, MO) and then all were exposed to Hyperfilm MP film (Amersham; Perkin-Elmer, Waltham, MA) at room temperature. Sections incubated with [^{3}H] muscimol were exposed for 14-28 weeks, those incubated with [^{3}H] flunitrazepam were exposed for 14 weeks, and sections incubated with [^{35}S] TBPS were exposed to film for 2-5 days. Film was then developed and fixed using a Kodak X-OMAT 1000A Processor (Kodak Co., Rochester, NY) for 1 minute.

[^{35}S]TBPS Non-specificity and Methodology

The [^{35}S]TBPS had unexpectedly high non-specific binding, particularly in comparison to the other two radioligands and to previous reports using this radioligand (Edgar & Swartz, 1990; Halonen et al., 2009; Kim et al., 2000; Leppa et al., 2005; Oh et al., 1999; Sah et al., 2002). Prior to using [^{35}S]TBPS I had attempted to use [^{3}H]TBOB, another radioligand for the pictoxin site (Kume et al., 1996; Milbrandt et al., 1996; Sakuri et al., 1994; Yagle et al 2003). The pilot using [^{3}H]TBOB this radioligand did show that picrotoxin successfully blocked its binding, although there was still some non-specific binding, which I expected to subtract out when I analyzed my films. I then conducted a full run with [^{3}H]TBOB, and included test slides

to develop throughout the exposure period to help me decide how long I should leave the film exposing to the slides. Instead of the 12 weeks indicated by the pilot, the film needed to be exposed to the [^{3}H]TBOB slides for almost 6 months before they were dark enough to analyze. Unfortunately, after I developed these films, I discovered that the radioligand was only negligibly blocked by picrotoxin in this full run, and that all of the labeling of the tissue sections looked homogenous. This indicated that the radioligand was non-specific. After this, I began to use [^{35}S]TBPS.

Over the next few months, I piloted this radioligand six times and altered my methodology to attempt to increase specificity. The steps that I altered included varying whether I fixed the tissue with paraformaldehyde as the first step, varying the time of pre-incubation buffers from between 5 minutes to 30 minutes and altering the make-up of the buffer so that it contained either 50 mM Tris-HCl only or included 120 mM NaCl and/or 1 mM EDTA. I also varied the time of incubation and recipe of the incubation buffer throughout these pilots, attempting incubating for 1 hour, 90 minutes, or 3 hours, and varying the incubation buffer so that it was either only 50 mM Tris-HCl, also included 120 mM NaCl and/or bovine serum albumin and bacitracin. In addition, I tested different concentrations of [^{35}S]TBPS, including 6, 3, 2, 1, and 0.5 nM concentrations. To be sure that one problem was not an inadequate amount of picrotoxin in the incubations testing for non-specific binding, I also included incubations with higher concentrations of picrotoxin (500 μM instead of 100 μM) and used another form of this compound, picrotoxinin, which also binds to the picrotoxin site. However, neither of these changes consistently affected non-specific binding. In the last of these pilots I had some success decreasing the homogeneity of the [^{35}S]TBPS binding. To try and increase the amount of

specificity further and determine why picrotoxin was not blocking binding, I contacted both the company that manufactures this radioligand (Perkin-Elmer, St. Louis, MO) and the PI of a lab that has extensive experience using this radioligand (Dr. Esa Korpi, University of Helsinki, Finland) to get advice and suggestions. Perkin-Elmer's support representative did not have methodology suggestions other than suggesting that different lot numbers of [^{35}S]TBPS might vary in binding specificity. Perkin-Elmer generously sent me two additional lots to pilot side-by-side with the lot I had already purchased and piloted. Dr. Korpi was extremely helpful and gave me useful suggestions and advice after reading my protocol and the numerous methodologies I had already tried, but unfortunately his laboratory had not used this radioligand in over a year, so he did not have recent information concerning specificity of new lots.

I conducted two final pilots in the weeks before completing my final [^{35}S]TBPS run, and by using EDTA to decrease the amount of total binding, always including 120 mM NaCl in pre-incubation and incubation buffers, and decreasing the concentration of radioligand, it appeared as if one of the lots in particular did bind specifically and with picrotoxin blocking much of the [^{35}S]TBPS binding. I followed this protocol and used the most specific lot to complete my full [^{35}S]TBPS autoradiography run, but unfortunately, even with an identical protocol, picrotoxin did not block the [^{35}S]TBPS as successfully in this run as it did in the previous pilot. Therefore, my results for this radioligand must be considered cautiously, as high non-specific binding complicates interpretation of the results. Even so, because this radioligand has been used successfully in the past (Edgar & Swartz, 1990; Halonen et al., 2009; Kim et al., 2000; Leppa et al., 2005; Oh et al., 1999; Sah et al., 2002), the films exposed to my tissue sections were not homogeneously lableled, and because I was able to block some [^{35}S]TBPS binding in some of

my pilots, these data may still add relevant information to the little that has been done comparing binding to the picrotoxin site across reproductive state and sex.

Data Analysis

Films were placed on a light box (0.35 Amps, 60 Hz; Knox Manufacturing Co., Wooddale, IL) and images were captured using a microscope digital camera (Roper Scientific Photometrics, Tucsan, AZ). Brain areas were determined using the Swanson (1998) atlas of the rat brain and images were analyzed using the Scion Image program to determine optical density values. Nonspecific binding was negligible for the [^{3}H] muscimol and [^{3}H] flunitrazepam slides, but was surprisingly high for the [^{35}S] TBPS slides, as discussed above. The non-specific binding present with [^{35}S]TBPS was close to the amount of specific binding and could not be subtracted from the optical density measurements while still leaving much possibility of detecting group differences. 1-3 brain sections were analyzed per subject for each brain region, and groups contained 4-8 subjects. Multiple optical density measurements were taken from each brain section bilaterally (Figure 8), the measurements were averaged within each brain region per hemisphere and then the two bilateral values were added together to get one measurement equivalent to the optical density of an entire bilateral brain region. These measurements were then averaged across brain sections from each subject for each brain region and the mean values from the groups were compared to determine possible differences. One-way ANOVAs for each site were used to determine group differences in density of receptor binding. Significance was determined at the $p < 0.05$ level and where the overall ANOVA showed significant group differences, Fisher's Least Significant Difference post-hoc tests was performed.

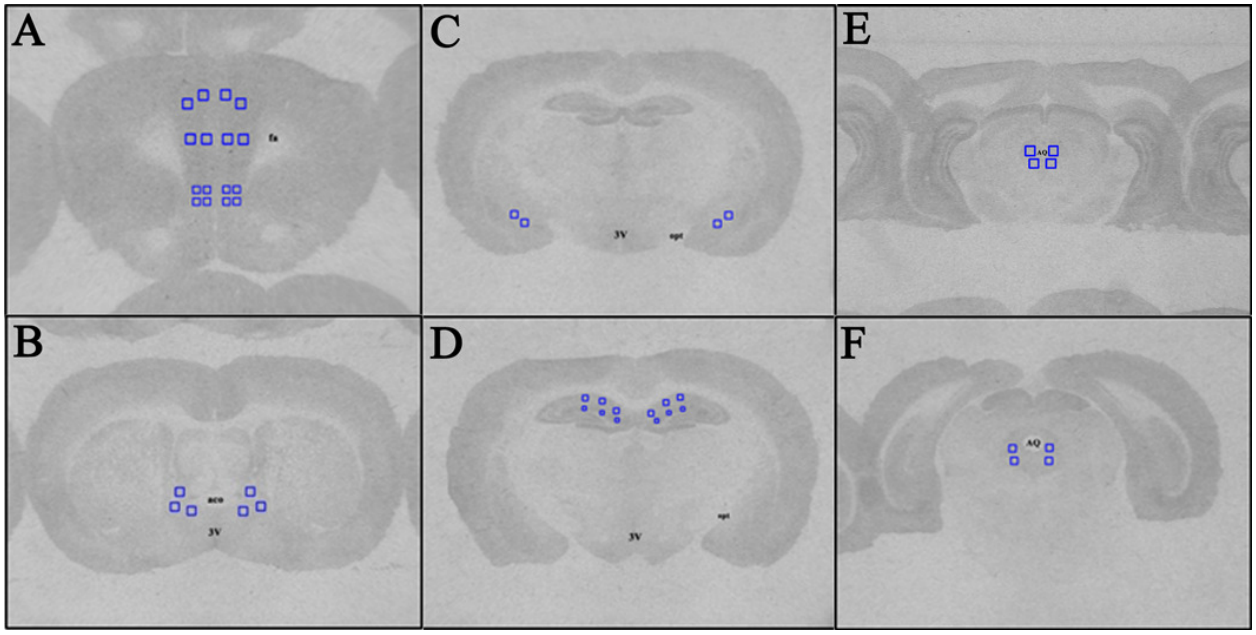

Figure 8. Representative autoradiograms of [^{3}H]FNP labeled coronal sections in the rat brain highlighting the areas sampled for optical density in each brain region. (A) mPFC, (B) BST, (C) CeA, (D) Hipp, (E) rPAG, (F) cPAG.

Results

There were significant main effects of group in the hippocampus regions such that diestrous virgins had significantly more [^{3}H]FNP binding in the DG ($F(3,24) = 3.891$, $p < 0.022$) and CA1 ($F(3,24) = 3.479$, $p < 0.032$) than did pregnant females or male rats; postpartum females were not significantly different than any other group (Figure 10). There were no group differences in [^{3}H]FNP binding in the mPFC, BST, CeA, rPAG, or cPAG (Figure 10). There were also no differences in [^{3}H]MUSC or [^{35}S]TBPS binding across groups in any of the brain regions examined (Figure 9, 11).

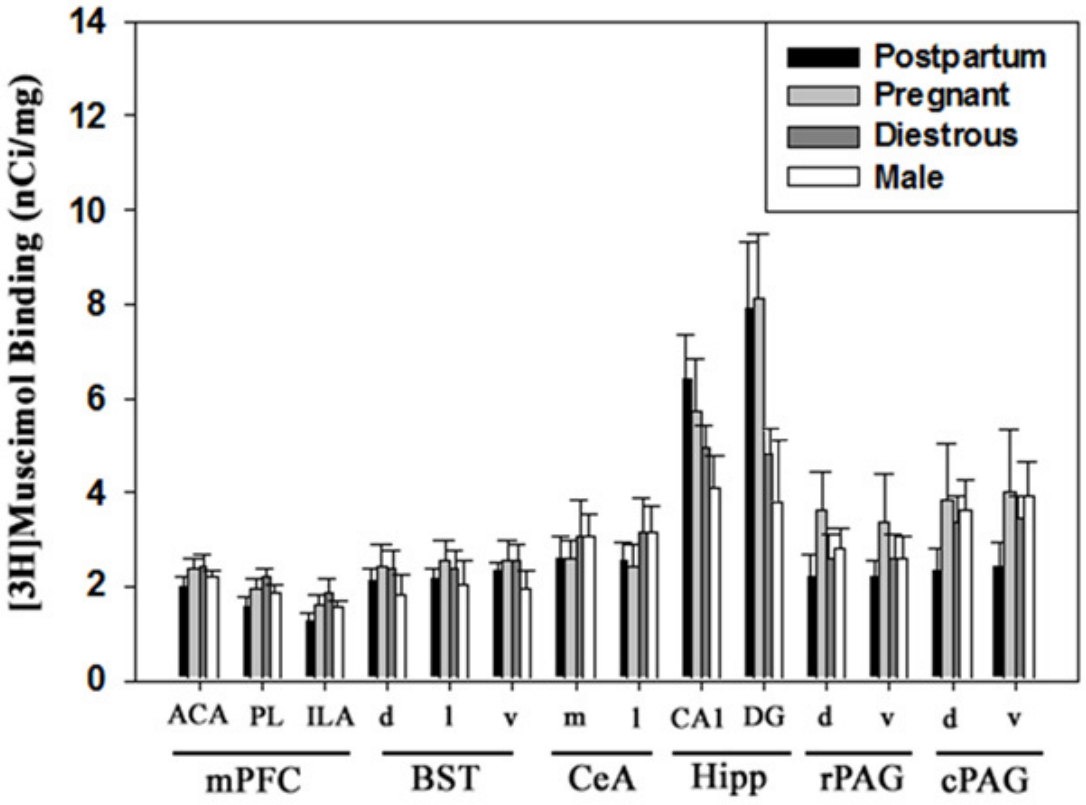

Figure 9. Relative optical density measurements of [^{3}H]Muscimol binding.

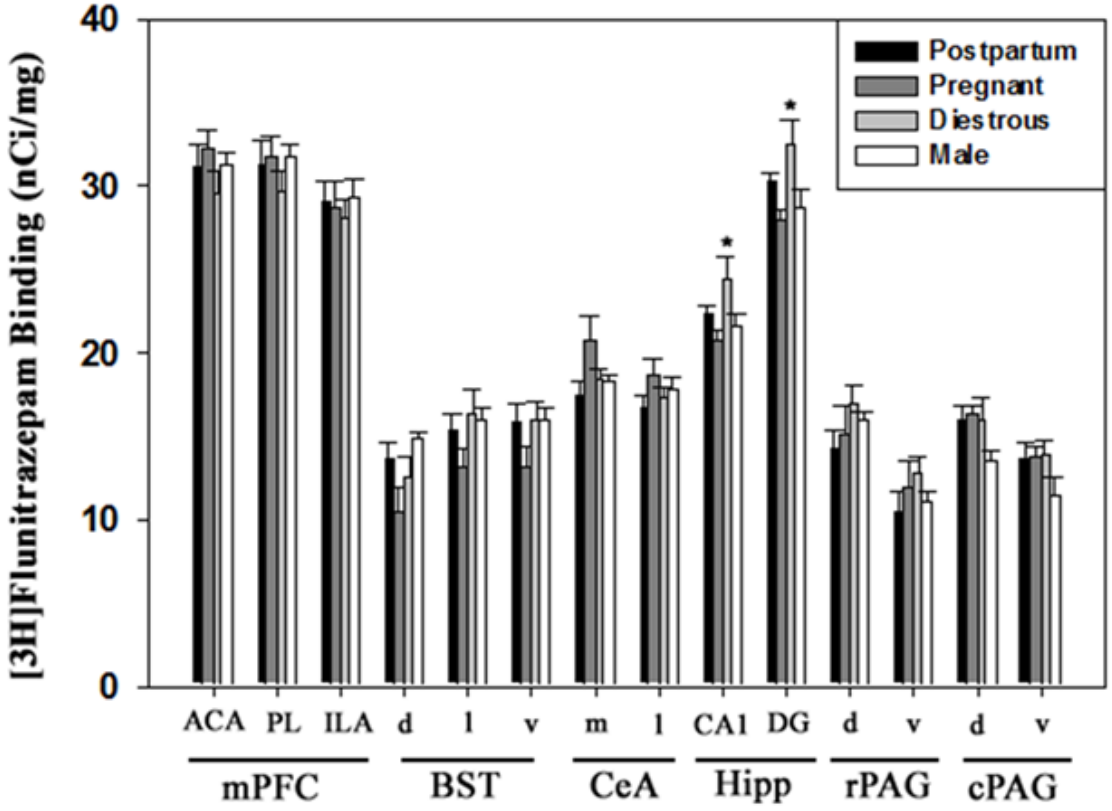

Figure 10. Relative optical density measurements of [^{3}H]FNP binding. * indicate significant differences with diestrous virgins having higher [^{3}H]FNP binding in CA1 and DG regions of the hippocampus than pregnant or virgin male rats.

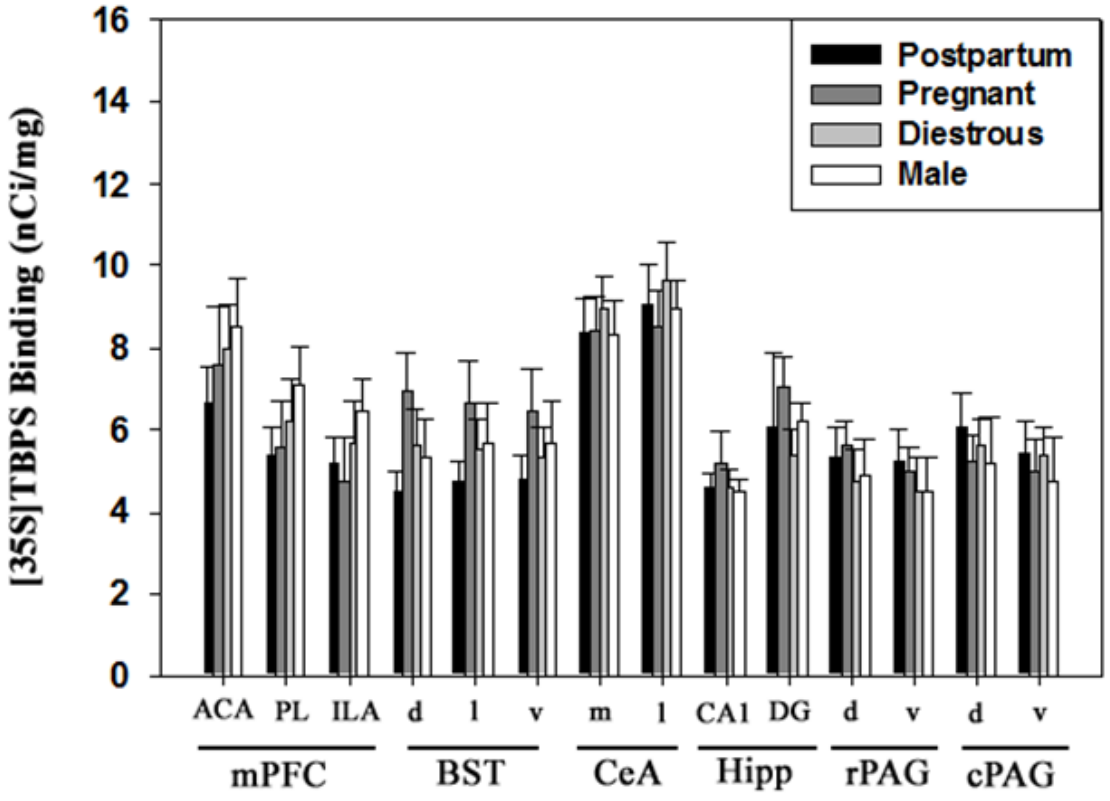

Figure 11. Relative optical density measurements of [^{35}S]TBPS binding.

Discussion

I have used autoradiographic analysis to investigate [^{3}H]MUSC, [^{3}H]FNP, and [^{35}S]TBPS binding to the $GABA_A$ receptor in six regions of the neural anxiety network of early postpartum, mid-pregnant, diestrous virgin, and sexually naïve male rats. I found that there was higher [^{3}H]FNP binding in the DG and CA1 regions of the hippocampus in diestrous virgins than pregnant or male rats, while postpartum females did not differ from any other group. There were no other group differences in [^{3}H]FNP binding, and no group differences in binding of

either [^{3}H]MUSC or [^{35}S]TBPS in any site examined, however conclusions from the [^{35}S]TBPS study must be made cautiously due to the methodological issues with non-specific binding that were mentioned above.

These results are somewhat surprising, as my first chapter led me to predict that any differences in binding to the $GABA_A$ receptor would be in binding to the picrotoxin site and that postpartum and diestrous females were likely to differ in [^{35}S]TBPS binding. However, I found no significant differences across these, or any other groups, in [^{35}S]TBPS binding. These negative results could be due to the non-specificity of the [^{35}S]TBPS radioligand, but if they are not, then these results indicate that the Chapter 1 picrotoxin site-specific effects I found are not driven by binding density differences. I also predicted a sex difference such that virgin males would have more [^{3}H]MUSC binding than virgin females as has been shown in the past possibly in relation to seizure susceptibility (Kokka et al., 1992), but this was also not the case.

From my Chapter 1 results, and the two previous studies that found no differences across reproductive state in [^{3}H]MUSC binding in the forebrain (Majewska et al., 1989) or within the cortex, hippocampus and cerebellum (Ferreira et al., 1989), I did not expect differences between postpartum and diestrous females in [^{3}H]MUSC binding and I did not find any. These previous studies homogenized the entire forebrain or used large tissue punches from the cortex, hippocampus, and cerebellum (Ferreira et al., 1989; Majewska et al., 1989). By examining binding in slide-mounted sections and within specific sub-nuclei of my brain regions of interest, it is possible that I would have been able to observe subtle differences masked by the use of large tissue samples (Ferreira et al., 1989; Majewska et al., 1989), if there were any. Even in Ferreira

and colleagues' study (1989), which was more site specific than Majewska and colleagues', there were many individual sub-nuclei included that either do not regulate anxiety behavior or are suspected to act in opposition with each other to mediate anxiety behavior. An even more obvious concern is including the entire forebrain in one homogenized sample because GABAergic systems regulate many diverse processes, including sex behavior, maternal behavior, aggression, thermoregulation, food intake, pain sensation, and locomotion (Arrati et al., 2006; De Almeida et al., 2005; Paredes & Ågmo, 1992). The diversity of GABAergic systems makes it difficult not only to predict where these overall differences in $GABA_A$ receptor binding are, but also which functions they might play a role in regulating.

Similar to my predictions for [^{3}H]MUSC binding, I did not expect and did not find [^{3}H]FNP binding to differ in these anxiety regulating brain regions between postpartum and diestrous females because I did not find many anxiogenic effects of antagonizing this site on light-dark box behavior, and none of the effects were specific to postpartum females (Chapter 1). Similarly, Ferreira and colleagues (1989) did not show differences in [^{3}H]FNP binding in large punches of the cortex, amygdala, hippocampus, or hypothalamus. However, what is surprising and inconsistent with these groups' anxiety behavior is that diestrous virgins had significantly higher [^{3}H]FNP binding in the DG and CA1 regions of the hippocampus in comparison to pregnant and male rats, but did not differ from postpartum females. There were no significant differences in [^{3}H]FNP binding in any other brain regions examined, which is consistent with the only other study to compare [^{3}H]FNP binding across reproductive states (Ferreira et al., 1989). Previous research indicates that diestrous female rats have higher [^{3}H]FNP binding in the frontal

cortex than do virgin males, and that this could be related to virgin females having different coping strategies to deal with stressors than do virgin males (Farabollini et al., 1996). In addition, thirty days after the end of avoidance conditioning, food deprivation, and a neophobia test, aged cycling females have higher [^{3}H]FNP frontal cortex binding than males, while male rats have higher [^{3}H]FNP binding in the hippocampus and striatum (Shephard et al., 1982). In other studies, [^{3}H]FNP binding in cortical tissue did not differ among male, oophphorhysterectomized, or late pregnant rats (McAuley et al., 1993), nor was binding different in the hippocampus or hypothalamus-preoptic area among male rats, females in each stage of the estrous cycle, or ovariectomized female rats (Wilson, 1992). While these studies are somewhat inconsistent, many confirm my findings of similar [^{3}H]FNP binding in many brain sites of postpartum, pregnant, diestrous, and virgin male rats. Shephard and colleagues (1982) did find that male rats had higher [^{3}H]FNP binding in the hippocampus than virgin females, the opposite of my finding, but this difference could be due to many methodological details. Shephard and colleagues' subjects had previously been tested in multiple other behavioral paradigms, which affects anxiety behavior (1982). They were also comparing males to cycling females without taking into account females' stage of the estrous cycle, while my female virgins were all in diestrus. Lastly, their subjects were all 200 days of age, which is considered mid-aged in rats (Frye et al., 2008) while my subjects were all young adults.

My finding that diestrous virgins have higher [^{3}H]FNP binding density in the DG and CA1 regions of the hippocampus in comparison to pregnant and virgin male rats, but no difference compared to postpartum females does not mimic these groups' patterns of anxiety

behavior. However, the suggestion that virgin females could have a different strategy for coping with stressors (Farabollini et al., 1996) could help to elucidate the possible functional significance of my findings. If indeed, the diestrous virgins in my study have more [^{3}H]FNP binding because they cope with stressors differently than male virgins, and presumably mid-pregnant females, then perhaps they are not different from postpartum females because they are using similar mechanisms to deal with stressors, but not necessarily to regulate anxiety behavior. Indeed, postpartum female rats show a dissociation between their anxiety behavior and their HPA axis activation (see Lonstein, 2005; Lonstein, 2007; Neumann, et al., 1998), so it is possible that postpartum females have a slight increase in [^{3}H]FNP binding in the hippocampus, putting their binding amount between that of diestrous virgins, and pregnant and male rats, and that this is related to their stress response but not anxiety behavior. Conversely, diestrous virgins could have more [^{3}H]FNP binding because they need more benzodiazepine binding sites in the hippocampus to respond to stressors, not necessarily through an anxiety response, and that this is a different mechanism than stress coping in mid-pregnant females or virgin males. Having a mechanism in place to cope with stressors that involves the hippocampus makes a great deal of sense as the hippocampus is an essential component of the negative feedback system for the HPA axis (Kim & Diamond, 2002; McEwen, 2000; Porter & Landfield, 1998).

Differences in [^{35}S]TBPS binding in brain regions regulating anxiety behavior would have been consistent with differences between postpartum and virgin females in their behavioral response to PTZ in the light-dark box (Chapter 1). If postpartum female brains were found to have fewer or more available picrotoxin sites than diestrous virgins, this difference could have been part of the mechanism underlying how anxiety behavior is naturally kept low during the

postpartum period. It also could have explained why PTZ had such a strong effect in postpartum females; if virgins naturally have many picrotoxin sites available and possibly active, but postpartum females have few if any activated picrotoxin sites, it would make sense that PTZ would have drastically changed the GABAergic activity in postpartum brains by acting at these unbound picrotoxin sites, while not significantly altering the already low GABAergic activity of diestrous virgins. Alternatively, if postpartum females had more picrotoxin binding sites, but naturally lower anxiety because of fewer endogenous picrotoxin agonists than diestrous virgins, PTZ could also have had a higher effect in dams than in diestrous virgins. Although total binding to the picrotoxin site may not be involved in creating this reproductive state behavioral difference, the picrotoxin site is clearly involved in some way given the Chapter 1 results. It is possible that a difference between groups in binding affinity to the picrotoxin site, as opposed to a difference in total binding, is associated with reproductive state differences in anxiety behavior. There is some past evidence that late pregnant rats have differential binding affinities to the GABA site compared to cycling virgins (Majewska et al., 1989) and there are also findings indicating that postpartum rats do not show differential binding affinities to the GABA, benzodiazepine, or picrotoxin sites (Ferreira et al., 1989; Majewska et al., 1989), however these studies used cycling virgins without first determining their stage in the estrous cycle, so it is likely that females in stages other than diestrus could have washed out possible group differences in affinity. In addition, male rats were not included in these studies (Ferreira et al., 1989; Majewska et al., 1989), so possible sex differences in $GABA_A$ receptor binding are also unknown. Therefore, it is possible that affinity differences in binding to the $GABA_A$ receptor contribute to differences in anxiety behavior, and differences in receptor subunit composition can alter binding affinity (Da Settimo et al., 2007; Korpi & Sinkkonen, 2006; Nutt, 2006; Sieghart et

al., 1999). In the next chapter, I examine the possibility that these groups differ in expression of $GABA_A$ receptor alpha subunits, which could relate to binding affinity to the picrotoxin site in regions mediating anxiety behavior and could also contribute to reproductive state and sex differences in anxiety.

Chapter 3: $GABA_A$ receptor subunit expression in the brains of postpartum, pregnant, diestrous virgin, and sexually naïve male rats

Introduction

GABA's ability to bind to and activate its A-type receptor is crucial for reduced anxiety during the postpartum period in rats. Chapter 1 demonstrates that inhibiting the $GABA_A$ receptor through the picrotoxin binding site strongly increases postpartum females' anxiety behavior in a light-dark box, while often not affecting that of diestrous virgins. These findings indicate that the picrotoxin site likely has a role specific to postpartum anxiety. In Chapter 2, I examined total binding to this site, as well as the GABA and benzodiazepine sites, and found no significant differences across reproductive states or sex in [^{35}S]TBPS or any other receptor site's binding. Due to the methodological issues with non-specific binding, these data must be considered cautiously, but if accurate, they indicate that total binding to the picrotoxin site is not a GABAergic mechanism contributing to reproductive state differences in anxiety. If total number of $GABA_A$ receptor binding sites is not contributing to differential anxiety behavior during the postpartum period, there must be another mechanism mediating GABA's role in this behavior. Binding sites on the $GABA_A$ receptor are formed by different subunits, and subunit composition greatly affects ligand-binding affinity (Da Settimo et al., 2007; Korpi & Sinkkonen, 2006; Sieghart et al., 1999). As noted previously in Figure 1, on the $GABA_A$ receptor, GABA's binding site is formed by the interface of the α and β subunits (Burt, 2003; Smith & Olsen, 1995), the benzodiazepine site is at the α-γ interface (Burt, 2003; Da Settimo, et al., 2007; Smith & Olsen, 1995), and the picrotoxin site is within the channel pore, and associated with the α subunit (Da Settimo et al., 2007; Korpi et al., 1997; but see Bell-Horner et al., 2000; Huang et

al., 2001). Changes in $GABA_A$ receptor subunit composition in the brains of postpartum female rats could be one mechanism contributing to their decreased anxiety, because the $GABA_A$ receptor could have decreased affinity for picrotoxin site agonists in postpartum brains, which would allow for higher $GABA_A$ activity, more inhibition, and lower anxiety. As the picrotoxin site is associated with the α subunit, even though specific affinities of α subtypes for picrotoxin agonists is not known (Huang et al., 2001; Kalueff, 2007), it is likely that whichever of these subunits confers a higher affinity to the picrotoxin binding site could be downregulated in postpartum brains in regions where GABA mediates anxiety, particularly in comparison to diestrous virgins, and that this could partially drive the postpartum anxiety reduction.

In non-parturient animals, differences in anxiety behavior are related to differences in $GABA_A$ receptor subunit expression, most often including expression of the α2, α3, and α4 subunits (Da Settimo et al., 2007; Nelokov et al., 2006; Nutt, 2006). For example, the α2 subunit mediates benzodiazepines' anxiolytic effects (Johnston, 2005; Löw et al., 2000; Nutt, 2006; Smith & Olsen, 1995), as mice with point mutations to the α2 subunit are insensitive to the benzodiazepine agonist diazepam's anxiolytic action (Löw et al., 2000). In addition, α2/α3 selective agonists are anxiolytic in rats tested in the elevated plus-maze, fear-potentiated startle, and conditioned suppression of drinking tests (Atack et al., 2006). Furthermore, the stimulation of specific neurons expressing α2/α3 subunits causes anxiety and fear (Korpi et al., 1997). Additionally, the α4 subunit of the $GABA_A$ receptor is upregulated when progesterone is withdrawn from male or female rats, and anxiety is also increased (Gulinello et al., 2002; Moran et al., 1998). These studies indicate that the α2, α3, and α4 subunits could be of particular importance for GABA's ability to regulate anxiety in non-parturient animals. However, little

work has investigated these $GABA_A$ receptor subunits specifically in the brains of postpartum females.

There have been a few investigations of $GABA_A$ receptor subunits across reproductive states or in models meant to mimic different reproductive states, but none comparing protein content of subunits in anxiety-related brain regions of Day 7 postpartum females compared to the more anxious diestrous virgins. $GABA_A$ receptor α2 mRNA expression is significantly reduced in the medial amygdala in post-weaning proestrous, primiparous females compared to proestrous, nulliparous controls (Byrnes et al., 2007). Conversely, α2 subunit mRNA expression is higher in the PAG of primiparous versus nulliparous proestrous females (Byrnes et al., 2007). In addition, on day 3 postpartum in comparison to day 20 of pregnancy in rats, there is an increase in the density of α2 or α3 subunits postsynaptically in neurons in the dorsomedial supraoptic nucleus (Koksma et al., 2005). Importantly, there is evidence that the picrotoxin site is associated specifically with the α2 subunit, and that the α4 subunit may also have affinity for picrotoxin and its ligands (Bell-Horner et al., 2000; Fradley et al., 2007). Furthermore, after progesterone withdrawal during pseudopregnancy or as a model of premenstrual anxiety, diestrous or ovariectomized female rats display increased anxiety and their α4 subunit content increases in both the hippocampus and amygdala through actions of the progesterone metabolite, 3α-5α-THP, on the $GABA_A$ receptor (Gulinello et al., 2003; Smith et al., 1998). Interestingly, progesterone withdrawal in female rats also increases picrotoxin-induced seizure activity, and this increase in picrotoxin's pro-convulsant effects is related to declining levels of the neurosteroid 3α,5α-THP (Moran & Smith, 1998). The α2 subunit also plays a role in mediating PTZ-induced seizures in male mice (Fradley et al., 2007). There is evidence to suggest that

picrotoxin binds equally well to α2β2γ2, α3β2γ2, and α6β2γ2 $GABA_A$ receptors, but may have some association with all isoforms of the alpha subunit (Bell-Horner et al., 2000). These past studies indicate that the α2, α3, and α4 subunits of the $GABA_A$ receptor are likely candidates for a role in regulating reproductive-state changes in GABA neurotransmission and postpartum anxiety behavior through differential expression in brain regions mediating anxiety.

Many brain regions modulate anxiety behavior through GABAergic neurotransmission, including the mPFC, BST, CeA, hippocampus, rPAG, and cPAG. It seems likely that if there are changes in $GABA_A$ receptor composition that occur during the postpartum period to contribute to dams' reduced anxiety and differences in the picrotoxin site, that these are likely areas where those potential changes could exist. In Chapter 1, I found that inhibiting the $GABA_A$ receptor through the picrotoxin binding site resulted in large anxiogenic effects, often specifically in postpartum females and not diestrous virgins, so it is possible that subunits associated with the picrotoxin site could be driving the postpartum reduction in anxiety and possibly increased sensitivity to picrotoxin site ligands, like PTZ. To determine whether there are differences in the α2, α3, and α4 subunits in these brain regions that could contribute to anxiety differences during the postpartum period, I have performed Western blot analysis of postpartum, mid-pregnant, diestrous, and virgin male rats' brains to determine the neural content of each of these three subunits in the mPFC, BST, CeA, hippocampus, rPAG, and cPAG. As of yet, the binding affinity of picrotoxin site ligands to bind to different α subunit subtypes is not confirmed (Bell-Horner et al., 2000; Fradley et al., 2007; Huang et al., 2001), but because these three subunits are known to be related to anxiety behavior, it is possible that they could do so through having different picrotoxin site affinities than other α subtypes. If these subunits have higher affinity for

picrotoxin site agonists, I hypothesize that postpartum females have higher concentrations of these subunits than the other groups, particularly diestrous females because in Chapter 1 I showed that postpartum females are much more sensitive to the effects of picrotoxin site agonists that are diestrous virgins. This hypothesis makes sense in the context of my theory from Chapter 2, which was that diestrous virgins, and presumably the other groups with higher anxiety that dams, might have a higher concentration of endogenous picrotoxin agonists and that this contributes to their higher anxiety. Therefore, if, as I hypothesize here, postpartum females do indeed have a higher concentration of alpha subunits that might have higher affinity for picrotoxin agonists, that would allow them to be more sensitive to PTZ, while also presumably having a low concentration of endogenous picrotoxin site agonists, so that their $GABA_A$ receptor system is still highly activated and contributing to their reduction in anxiety.

Methods

Subjects

Subjects were housed and treated as described in Chapter 2.

Tissue collection and homogenization

On a day of diestrus (n = 10), 10 days (±1 day) after insemination (n = 10), 7 days (±1 day) after parturition (n = 10), or after 75 days of age for males, subjects were anesthetized with CO_2 for < 1 minute, and then rapidly decapitated using a guillotine. Their brains were immediately removed and placed on dry ice, then stored at -80^{o}C until tissue punching. Half millimeter thick sections through the mpfc, BST, CeA, hippocampus, cPAG, and rPAG were cut on a cryostat at -20^{o}C and then tissue punched bilaterally with an 18-gauge stainless-steel tube. Punches through the mpfc were from ~ +3.2 mm to ~ +2.2 mm from bregma (approximately

corresponding to plates 8-10 from Swanson, 1998) and were between the midline and the anterior forceps (fa), from ~ 1 mm ventral from the dorsal surface of the brain to ~ 5 mm. These punches included the anterior cingulate cortex (ACA), the prelimbic cortex (PL), and the infralimbic area (ILA). BST punches were from ~ 0.0 (bregma) to ~ -1.00 mm from bregma (approximately atlas plates 17-23), and included portions of all BST subdivisions. CeA tissue punches began ~ -1.3 mm from bregma and ended ~ -2.30 mm from bregma (corresponding to slightly anterior to atlas plates 24 to 28), and the medial/lateral and dorsal/ventral location of the CeA were determined by utilizing the stria terminalis (st) and external capsule (ec) fiber bundles. Punches through the hippocampus were collected serially (1 mm per punch) from ~ -2.45mm to ~ -6.45 mm from bregma (approximately atlas plates 28 to anterior to plate 40) and included both the dorsal and ventral hippocampus. PAG punches were also collected serially (1 mm per punch) from ~ -4.45 to -6.06 mm from bregma (approximately plates 34 to 39) for the rPAG and from ~6.60 to -8.45 mm from bregma (approximately from just caudal to plate 41 to anterior to plate 47) for the cPAG. (See Figure 12). After sectioning and punching, tissue was pooled by brain region and group to assure that there would be enough sample from the smaller brain regions, with five subjects from each group pooled together and 4 pools total per group, homogenized and stored at -80^{o}C until all tissue was collected.

Tissue was homogenized in buffer (10 mM Tris, 10% glycerol, 400 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.4) with protease inhibitors (1:10 dilution; 2 mM AEBSF, 1 mM EDTA, 130 μM Bestatin, 14 μM E-64, 1 μM Leupeptin, 0.3 μM Aprotinin; Santa Cruz Biotechnology, Santa Cruz, CA). Samples were then centrifuged for 1 hour at 4^{o}C at 15,000 rpm to sediment cellular debris and nuclei. Following centrifugation, supernatants were collected, samples were frozen, and then stored at -80^{o}C until further processing.

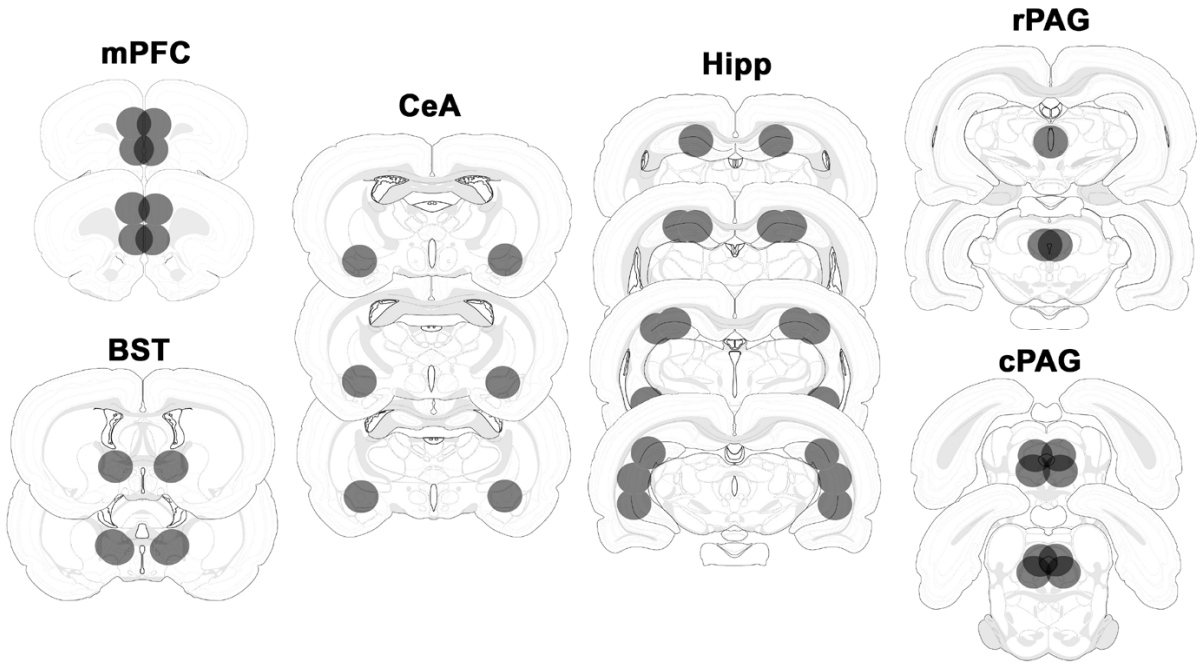

Figure 12. Schematic representation of the regions of the mPFC, BST, CeA, Hipp, rPAG, and cPAG included in the tissue punches for Western blot analyses (indicated by shaded areas).

Western Blot Analysis

Samples from each brain region from each group were assayed by Western blot analysis for detection of $GABA_A$ receptor subunits α2 (51 kDa), α4 (67-70 kDa), and α3 (53-55 kDa) proteins. One hundred fifteen μg of total protein from each pooled sample was gel electrophoresed on 10% polyacrylamide gels containing 1% SDS and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). A high amount of protein was loaded to attempt to ensure that there would be enough protein so that bands would be easily visible because some alpha subunits of the $GABA_A$ receptor are in very low abundance in some regions of the brain (Smith et al., 2006). Membranes were then washed three times for 10

minutes each in TBS-T (TBS containing 0.05% Tween-20) and blocked in TBS-T with 5% nonfat dry milk for 1 hour at room temperature. Membranes were then incubated in a goat polyclonal antibody against $GABA_A$ receptor subunit α2 (1:1000; sc-7350; Santa Cruz Biotechnology, Santa Cruz, CA; Foley et al., 2003; Guerra-Azaiza et al., 2008) in TBS-T and 0.02% sodium azide overnight at 4°C. Following the incubation, membranes were washed three times for 10 minutes each in TBS-T, incubated in a peroxidase-conjugated rabbit anti-goat secondary antibody (1:80,000, Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature, and then rinsed in TBS-T three times for 10 minutes each. Detection of immunoreactive bands was accomplished using an enhanced chemiluminescence kit (Western blot luminol; Santa Cruz Biotechnology, Santa Cruz, CA), after which membranes were exposed to film (Blue Sensitive X-ray film, Laboratory Products Sales, Rochester, NY) for between 30 seconds and 5 minutes, depending on signal strength, then developed and fixed using a Kodak X-OMAT 1000A Processor (Kodak Co., Rochester, NY) for 1 minute.

Following immunoblot detection of the α2 subunit, membranes were then stripped and reused to examine the levels of α4 and α3 protein in these samples, and then a final time to probe for GAPDH levels to control for the total amount of protein loaded. Membranes were rinsed twice for a few seconds each time in TBS-T, and then stripped in stripping buffer (2% sodium dodecyl sulfate, 62.5 mM Tris HCl, 100 mM 2-mercaptoethanol, H_2O, pH 6.7) for 3 hours in a water bath at 70°C. After stripping, membranes were washed four times for 10 minutes each in TBS-T at room temperature, blocked again in TBS-T with 5% nonfat dry milk for 1 hour at room temperature, and then reprobed for the α4 subunit protein by incubation in a goat polyclonal antibody against $GABA_A$ receptor subunit α4 (1:1000; sc-7355; Santa Cruz Biotechnology, Santa Cruz, CA; Sanna et al., 2003) in TBS-T and 0.02% sodium azide overnight at 4°C.

Following the incubation, membranes were washed three times for 10 minutes each in TBS-T, incubated in a peroxidase-conjugated rabbit anti-goat secondary antibody (1:80,000, Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature, then rinsed three times for 10 minutes each time. Immunoreactive bands were again detected using Western blot luminol (Santa Cruz Biotechnology), and then exposed to film as described above.

Membranes were then stripped a second time in stripping buffer, washed, blocked again, and reprobed with a rabbit polyclonal antibody against $GABA_A$ receptor subunit α3 protein (1:1000; AB5594; Millipore, Billerica, MA) overnight at 4°C. Following this incubation, membranes were washed, incubated in a peroxidase-conjugated goat anti-rabbit secondary antibody (1:1,000, Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature, then rinsed. Immunoreactive bands were detected and membranes exposed to film as previously described. Finally membranes were stripped and reprobed as described above with a mouse polyclonal antibody against GAPDH (1:500; MAB374; Millipore, Billerica, MA) and a peroxidase-conjugated rabbit anti-mouse secondary (1:80,000; Sigma-Aldrich, St. Louis, MO).

Preabsorption Controls and Methodological Considerations

As is represented in Figure 13, my Western blots had a relatively high background, and in addition had a relatively high number of what are presumably non-specific bands within each lane. Although I analyzed distinct bands at the specified kDa weights for my subunits of interest, these extra bands are concerning. These bands reportedly could indicate either partially degraded portions of the subunits I am interested in or they could be splice variants of the subunits, but they could also indicate non-specific labeling. To attempt to confirm the specificity of my primary antibodies, and to confirm the kDa weights of my subunits, I performed

preabsorption controls to attempt to block all specific labeling by adding the actual peptide to my primary antibody solutions before applying them to my membranes. This should have confirmed which band was the specific band for these subunits. I did not realize until notified by members of my committee that it is common to use a 10-fold increase of peptide in comparison to the amount of primary antibody used. The many reference articles I read often did not specify what controls they had performed for the antibodies they used for their Western blots, and if they did specify that preabsorption controls were performed, they did not report how much peptide was used. I was unaware until this past summer that I needed to do preabsorption controls and when I did perform these controls, I followed the primary antibodies' manufacturers' instructions, which specified to use equal amounts of peptide and primary antibody. I attempted the preabsorption controls for all three of my antibodies on stripped pilot membranes first, and did not see a noticeable reduction in labeling. I then attempted to preabsorb my antibodies again using different stripped membranes so that I would be able to compare the preabsorbed films with the already presumably specifically labeled films. Again, I saw no reduction in labeling. At this point, I discussed the situation with my advisor and because we had heard instances of this happening with other primary antibodies and of people still publishing their results even without preabsorption controls being successful, neither of us realized that we were dealing with a big problem with my study. In addition to discussing this issue with my advisor, I also talked to someone who has lots of experience with doing Western blot analyses, and this person also did not know what to make of the failed preabsorption controls, but did not seem to think this was a problem with my study. Unfortunately, it seems that I was not asking the right people, or not asking enough people's opinions, and am now learning from that oversight.

From the advice my dissertation committee has now offered, I realize that I did not use enough peptide in my preabsorption controls to adequately block my primary antibodies, so at this point I do not know whether my primary antibodies specifically labeled my Western blots or whether they were non-specific.

Image and Data Analysis

Films were placed on a light box (0.35 Amps, 60 Hz; Knox Manufacturing Co., Wooddale, IL) and images were captured using a microscope digital camera (Roper Scientific Photometrics, Tucsan, AZ). Image J was then used to determine the integrated density (area of band x mean optical density) of the immunoreactive bands (see Figure 13). All α2, α3, and α4 subunit integrated density measurements were standardized using their corresponding GAPDH integrated density measurements. One-way ANOVAs for each subunit for each brain site were used to determine group differences in GAPDH-standardized integrated densities of protein content. Significance was determined at the $p < 0.05$ level and where the overall ANOVA showed significant group differences, the Fisher's Least Significant Difference post-hoc test was performed to compare individual pairs of groups.

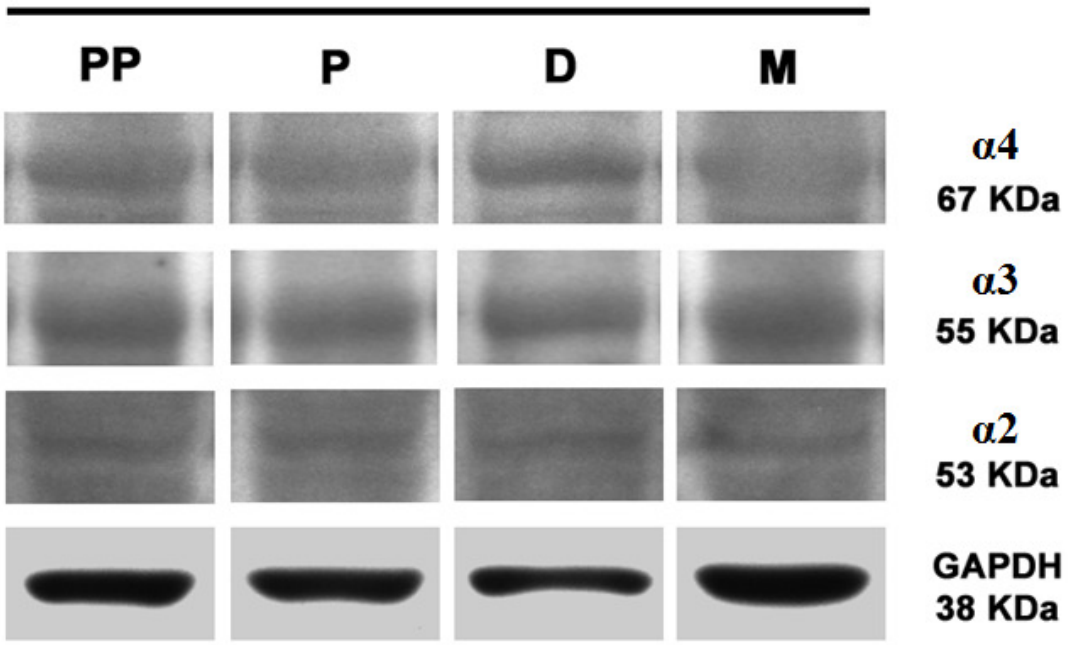

Figure 13. Representative Western blot showing bands for the α2, α3, and α4 subunits and loading control protein (GAPDH). PP = day 7 postpartum, P = mid-pregnant, D = diestrous, M = male.

Results

There was a significant main effect of group such that males had significantly more α2 subunit content in the rPAG than did either postpartum or diestrous females; pregnant females were not significantly different than any other group ($F(3,12) = 5.27$, $p = 0.015$; Figure 18). There were no significant differences in α3 or α4 content in the rPAG, nor were there significant differences across the groups in α2, α3, or α4 subunit content in any of the other brain regions examined (mPFC, BST, CeA, Hipp, cPAG, Figures 14-17, 19).

It is important to note that these results must be considered cautiously because preabsorption controls for these antibodies did not block any labeling, indicating that the labeling could have been non-specific.

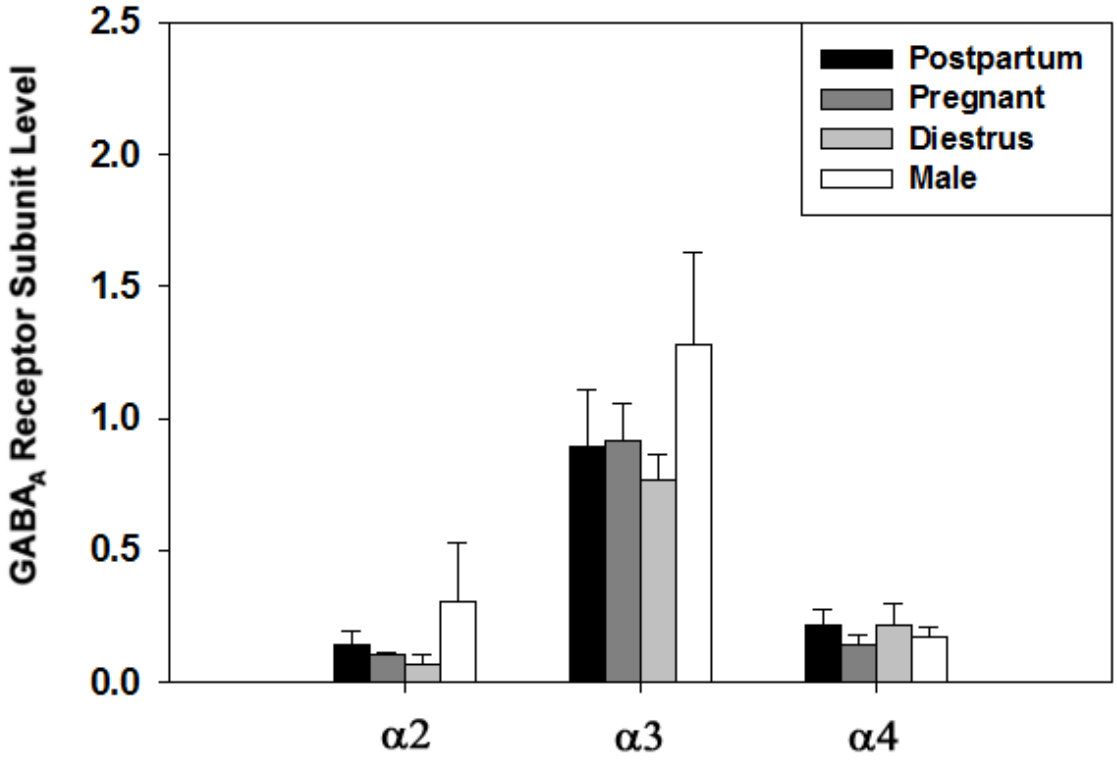

Figure 14. Relative optical measurements of α2, α3, and α4 subunit concentrations in the mPFC.

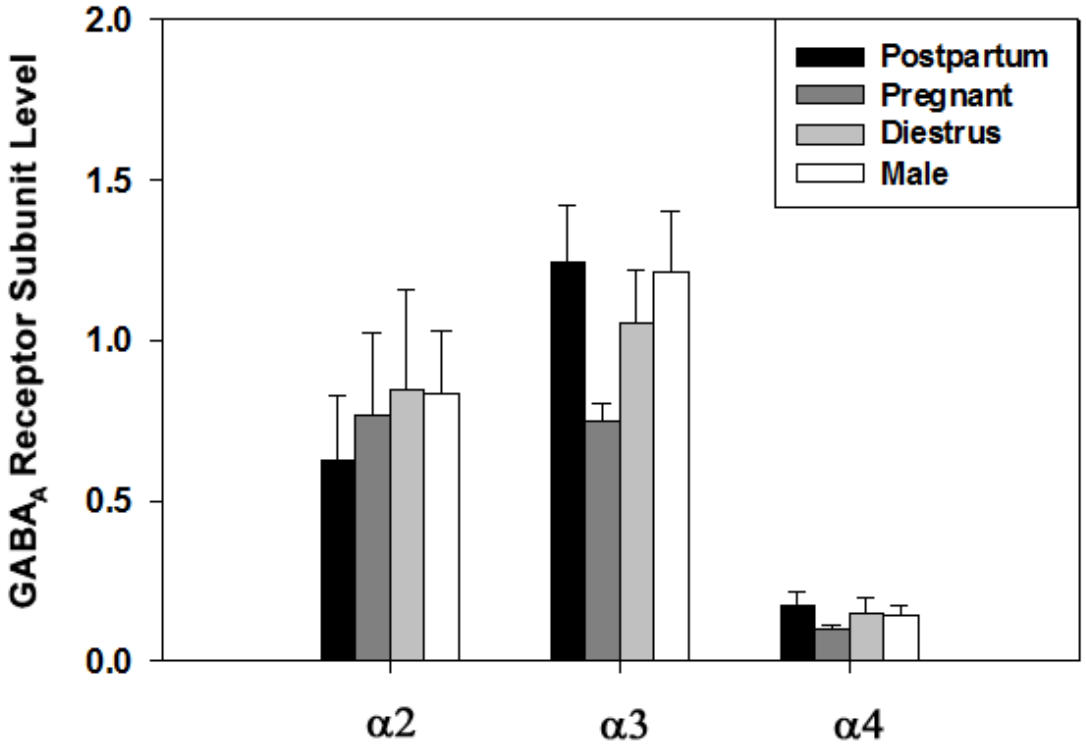

Figure 15. Relative optical measurements of α2, α3, and α4 subunit concentrations in the BST.

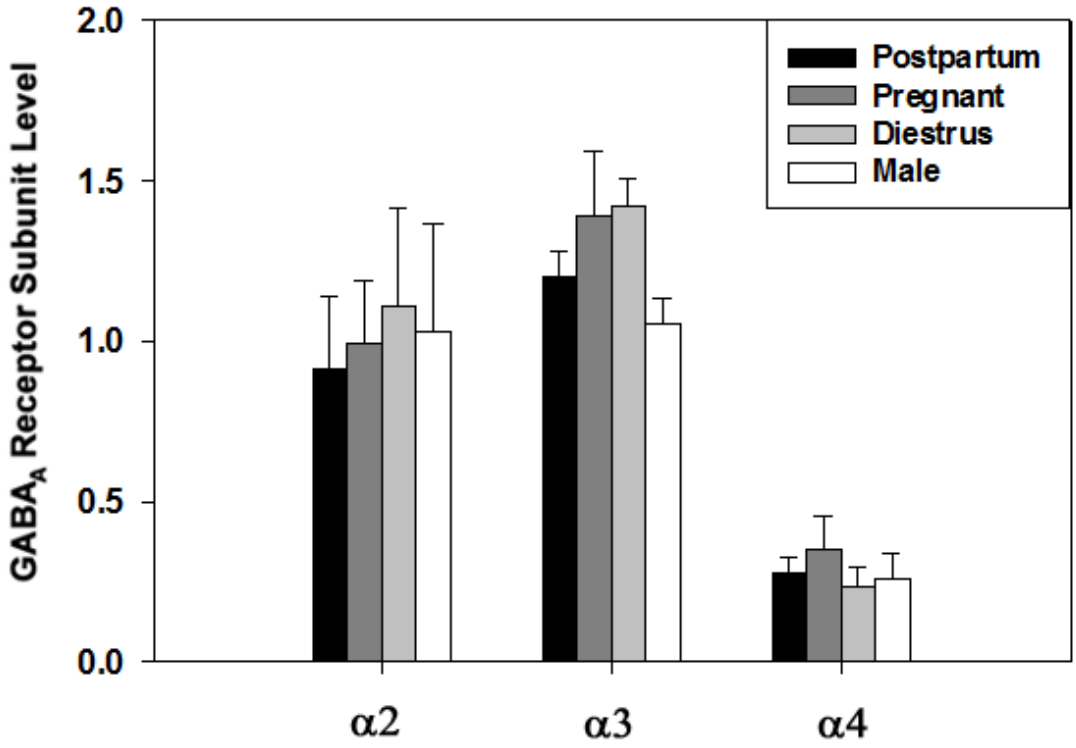

Figure 16. Relative optical measurements of α2, α3, and α4 subunit concentrations in the CeA.

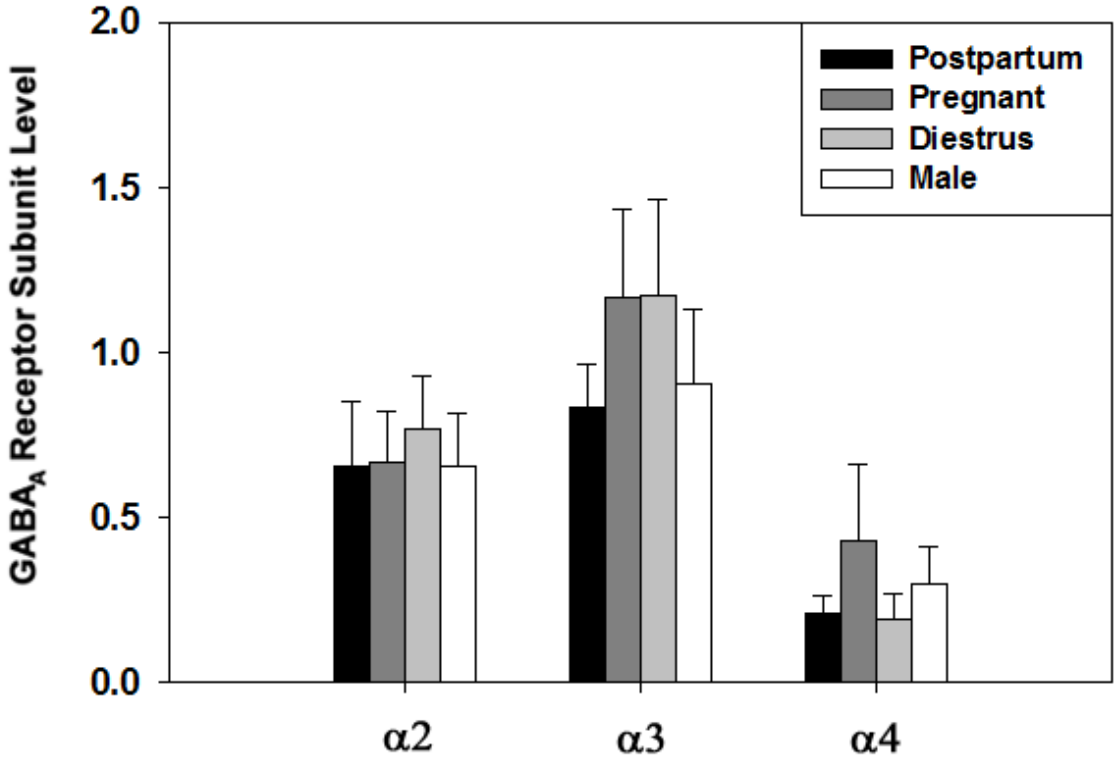

Figure 17. Relative optical measurements of α2, α3, and α4 subunit concentrations in the Hipp.

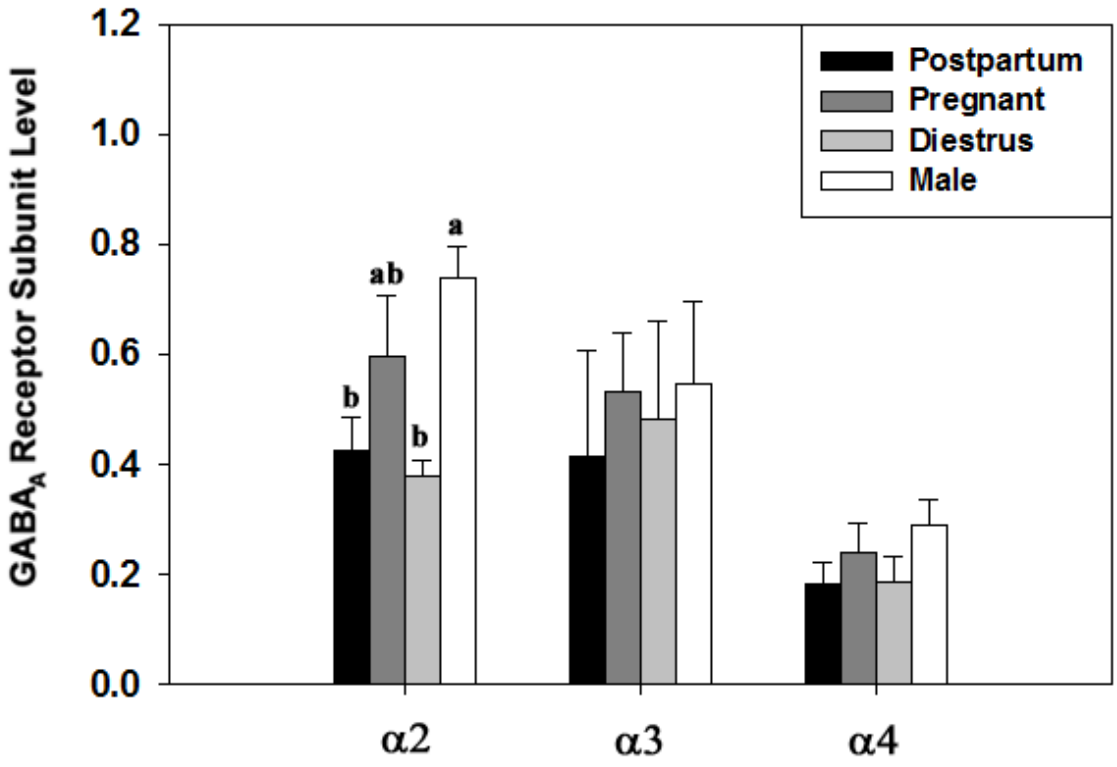

Figure 18. Relative optical measurements of α2, α3, and α4 subunit concentrations in the rPAG. Letters above bars indicate significant group differences.

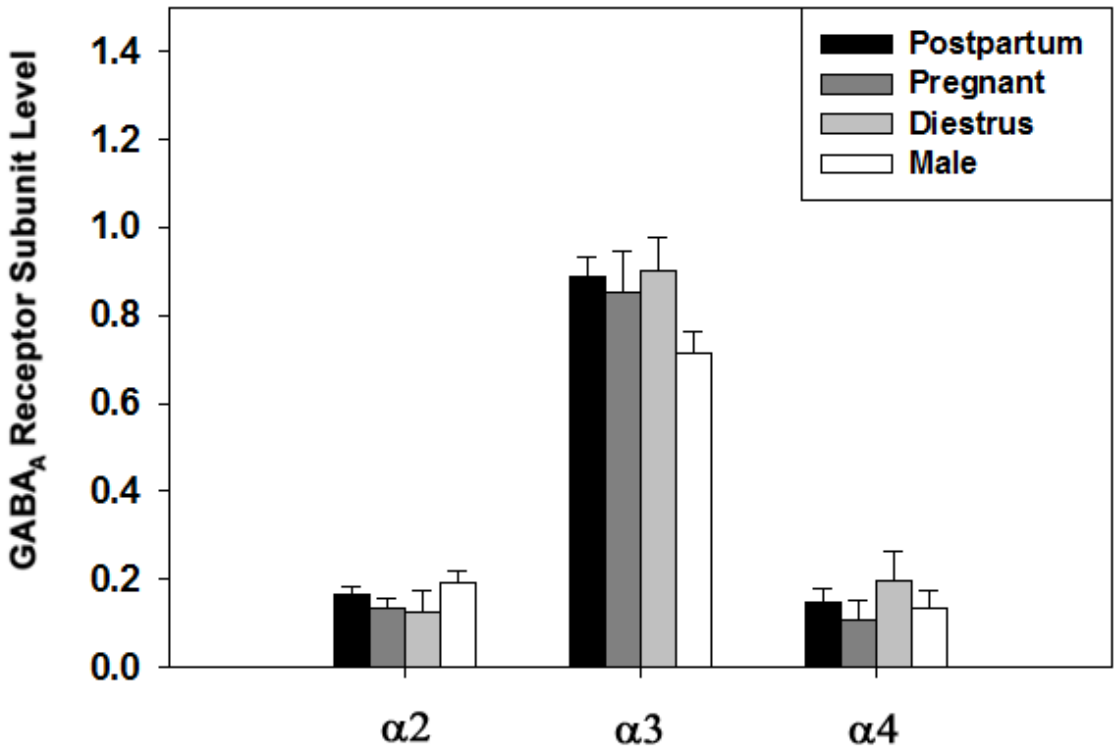

Figure 19. Relative optical measurements of α2, α3, and α4 subunit concentrations in the cPAG.

Discussion

I have used Western blot analysis to investigate the concentration of the α2, α3, and α4 subunits of the $GABA_A$ receptor in six regions in the brains of postpartum, pregnant, diestrous virgin, and male rats. I found that content of the α2 subunit is significantly lower in the rPAG in early postpartum and diestrous female rats than in sexually naïve male rats, while content in the brains of Day 10 pregnant females did not significantly differ from any other group. I also found

that the α2 subunit did not differ across groups in the mPFC, BST, CeA, Hipp, or cPAG, nor did the α3 and α4 subunits differ across groups in any of the brain regions examined.

Because of the methodological issues noted above, these results must be considered skeptically until the completion of further attempts at preabsorption controls for these primary antibodies. My next step will be to do further preabsorption controls to determine whether these antibodies are specific. I will first attempt a 10-fold higher amount of peptide than primary antibody, and if I see either no reduction in labeling or a reduction but not complete blocking of bands, then I will perform these controls again using a 50-fold higher concentration of peptide than primary antibody so that I will be completely sure that if the peptides can block the primary antibodies, that they will be concentrated enough to do so. If any of my new preabsorption controls block primary antibody labeling of my blots, I will take this as confirmation that those blocked primary antibodies are specifically labeling for my alpha subunits of interest and that their data are valid. Along with my new preabsorption controls, I will also perform a no-primary control to determine whether my secondary antibodies or any other step in my Western blot procedure is causing non-specific labeling. If one of the steps aside from the primary antibodies is causing the non-specific labeling, I will look into other products that could be substituted for that step, and either I, or someone else I have trained in the lab, will re-pilot to determine whether we can eliminate the non-specific labeling. If we can, my leftover samples could be re-run and re-analyzed using this new protocol. If one of the steps besides the primary antibody step is not causing the non-specific labeling, or if the step that is causing it cannot be fixed, this project may have to be abandoned, or at least put on hold in hopes that new primary antibodies with more specificity or new products for the other steps are developed.

If the results of the analyses are reliable, one of the simplest explanations for the possible α2 subunit content difference I found, taking into account the α2 subunit's known involvement in anxiety behavior, is that there could be a sex difference in the GABAergic system underlying anxiety behavior between virgin males and diestrous females. As discussed previously, male rodents with low testosterone, such as my sexually naïve male group, tend to display similar levels of anxiety behavior as virgin female rodents (Mora et al., 1996; Toufexis, 2007; Wilson et al., 2004; Zuluaga et al., 2005). Therefore, it would seem that if the α2 subunit regulates anxiety behavior in the rPAG, that virgin males and females should have similar levels of α2 subunit, but they might not; I found that males had more α2 subunit than female virgins, indicating a possible sex difference in how the rPAG regulates anxiety. In addition, early postpartum female rats are most often found to have lower anxiety behavior than diestrous virgins (Fleming & Luebke, 1981; Lonstein, 2005; Smith & Lonstein, 2008; Toufexis, 2007; Toufexis et al., 1999), suggesting that they must also have lower anxiety behavior than sexually naïve male rats, as these males have similar anxiety behavior to diestrous virgin females. As a result, postpartum females should have differential subunit expression from both female and male virgins, if indeed these subunits are driving anxiety behavior, but postpartum females only possibly had lower α2 content in the rPAG in comparison to virgin males, and possibly similar levels as diestrous virgins. Finally, findings on anxiety during pregnancy in rats are controversial, but many studies indicate that anxiety in mid-pregnant rats is similar to virgin rats (De Brito Faturi et al., 2006; Neumann et al., 1998), indicating that pregnant females should have subunit expression similar to that of diestrous virgins and male virgins, again, if these subunits are involved in anxiety behavior. However, these predictions are not what I found in the rPAG, as subunits' expression in pregnant females potentially was not significantly different than either virgins or postpartum

females. As α2 subunit content may not differ in females in correspondence with their differences in anxiety behavior, perhaps the reproductive state difference in anxiety, and in responsiveness to PTZ, is not mediated through rPAG α2 content. It is possible that this potential α2 subunit difference actually indicates that the sexes are regulating anxiety through different mechanisms in the rPAG. Perhaps the amount of α2 subunit in the rPAG differs between male rats of differing anxiety levels, while it is not involved in anxiety differences between female rats of differing hormonal levels and reproductive experiences. In fact, expression of the α2 gene is higher in the rostral and caudal PAG in male rats that display high anxiety-related behavior in the elevated plus-maze in comparison to males that exhibit lower anxiety-related behavior (Nelovkov et al., 2006). Therefore, it may be that α2 subunit content in the rPAG is related to individual differences in anxiety within male, but not female, rats.

The rPAG is sexually dimorphic and involved in numerous behaviors, including anxiety. Both GABAergic and serotonergic receptors in the rPAG mediate anxiety and panic-like behavior in male rats (Graeff et al., 1993; Motta & Brandao, 1993). One example of sex differences in this region is that female rats exhibit a higher number of cells in the rPAG that project to the nucleus paragigantocellularis in the brainstem than do male rats, in response to sex (Normandin & Murphy, 2008). The rPAG is also involved in regulating pain and does this in a sexually dimorphic way, as males are much more responsive to the antinociceptive effects of opioid agonists infused in this region than are female rats (Krzanowska et al., 2000; Krzanowska & Bodnar, 2000; Loyd & Murphy, 2009), and this sex difference most likely occurs through the removal of more tonic GABA inhibition in the rPAGs of males than in females (Loyd & Murphy, 2009). As the rPAG is known to be involved in many behaviors, including anxiety and the other behaviors mentioned here, it is also important to note that the possible difference in

expression of the $GABA_A$ receptor α2 subunit that I found in this region could be unrelated to anxiety behavior and instead be relevant to a different, also presumably sexually dimorphic, behavior modulated by the rPAG. However, because the α2 subunit is so heavily involved in anxiety behavior (Burt, 2003; Burt, 2005; Gee et al., 2010; Korpi & Sinkkonen, 2006; Rudolph & Möhler, 2004; Rudolph & Möhler, 2006), and the rPAG has regularly been studied for its role in anxiety in male rats (Graeff et al., 1993; Motta & Brandao, 1993), it seems likely that this potential difference would be related to anxiety. Indeed, as mentioned above, it is possible that there is a sex difference in how the rPAG is involved in anxiety behavior, and specifically how expression of the α2 subunit in the rPAG mediates anxiety behavior within males and females. The possibility that female and male rats may have differing GABAergic mechanisms that contribute to anxiety differences within the sexes may seem unnecessarily complicated, however, there is at least one potential explanation that could account for different $GABA_A$ receptor subunits being relevant to anxiety in one sex and not the other: neurosteroids.

Neurosteroids are neuroactive metabolites of steroid hormones, such as the progesterone metabolite 3α-hydroxy-5α-pregnan-20-one (3α, 5α-THP or allopregnanolone), that can be synthesized in both the peripheral and central nervous systems (Maguire & Mody, 2009; Mostallino et al., 2009; Sanna et al., 2009). Neurosteroids bind to the $GABA_A$ receptor and enhance neuronal inhibition (Mostallino et al., 2009), and $GABA_A$ receptors that contain the δ subunit in place of a γ subunit are particularly sensitive to the effects of neurosteroids (Maguire & Mody, 2009; Mostallino et al., 2009). In addition, expression of $GABA_A$ receptor δ and γ2 subunits changes in response to fluctuating hormone levels, especially those during pregnancy, and this regulation of $GABA_A$ receptor subunits has been suggested to be mediated by

neurosteroids (Concas et al., 1998; Lovick et al., 2005; Maguire & Mody, 2009; Mostallino et al., 2009; Sanna et al., 2009). The α2 subunit is not responsive to neurosteroids, so perhaps female rats' anxiety behavior is modulated by a different subunit that is capable of responding to neurosteroids so that their brains are ready to respond to hormonal fluctuations (Burt, 2003; Rudolph & Möhler, 2004). It is possible that females have less α2 subunit in their rPAG because they have a higher amount of subunits that are sensitive to neurosteroids, such as the δ and γ2 subunits. The δ and γ2 subunits are most often found in combination with α1 and β2/β3 subunits (Benke et al., 1991) in $GABA_A$ receptors, so maybe postpartum and diestrous females have potentially downregulated α2 in the rPAG in favor of more α1 subunits to be in combination with γ2 or δ containing receptors. In addition, it is possible that mid-pregnant females had α2 subunit expression in the rPAG that was possibly not significantly different than that of any of the other groups because their brains are already responding to the large fluctuations in hormones that occur during pregnancy, and particularly to increases in neurosteroid concentrations (Maguire & Mody, 2008). It is possible that for mid-pregnant females to mediate their anxiety at this time they are beginning to need more $GABA_A$ receptors that do not respond so readily to neuroactive hormones. In fact, both the δ and γ2 subunits decrease abundance in some brain regions throughout pregnancy (Maguire & Mody, 2008) so perhaps by mid-pregnancy, female rats have already downregulated these subunits, possibly in favor of more receptors including the α2 subunit. It would seem particularly advantageous if anxiety in female rats could be partially modulated by increasing and decreasing subunits that are responsive to neurosteroid fluctuations, whereas male rats would not naturally need this ability.

As there may not be alterations in these subunits' content in these brain regions, other than the possible α2 subunit increase in the rPAG, in sexually naïve males, differences in

concentration of α2, α3, and α4 subunits in these regions may not contribute to the sex and reproductive state differences in anxiety behavior between these groups. If this is true or false, it is still possible that there are other subunits whose differential expression in these regions does contribute to the regulation of anxiety differences across sex and reproductive state, so further studies of these possibilities are still necessary. Mice that are heterozygous for the γ2 subunit gene exhibit increased anxiety in the elevated plus-maze, light/dark choice, and free-choice exploration tests (Crestani et al., 1999), making this subunit another likely candidate to differ across groups that display varied anxiety behavior. There is also some evidence indicating the α1 subunit in anxiety behavior (Burt, 2003; Korpi & Sinkkonen, 2006; Rudolph & Möhler, 2006), though findings are not as conclusive as with the α subunits I investigated. In addition, possible differences in $GABA_A$ receptor subunit expression would not preclude the likely possibility that the GABAergic system could still be different in other ways in these brain regions between these groups, which could contribute to differences in anxiety behavior. For example, again, it is likely that postpartum females have higher GABA release in one or more of these regions in comparison to groups with higher anxiety, as litter contact both decreases anxiety and increases cerebrospinal fluid concentrations of GABA in postpartum females (Lonstein, 2005; Neumann, 2003; Qureshi et al., 1987; Smith & Lonstein, 2008), making this one additional likely mechanism for reducing anxiety during the postpartum period. The potential difference in the α2 subunit in the rPAG could be related to differences in the picrotoxin binding site, but it is also possible that the subunits I investigated are not the most relevant for the picrotoxin site or that these are not the alpha subunits that have differential affinity for picrotoxin site ligands. More research is required to examine whether changes in α2 content in the rPAG could be related to differences in the picrotoxin site. In addition, there is a

need to investigate which subunits are the most important for picrotoxin site binding to the $GABA_A$ receptor and to determine in which brain regions differences in the picrotoxin site could influence anxiety in postpartum female rats.

General Discussion

My dissertation work focused on investigating potential GABAergic mechanisms that could underlie the differences in anxiety between early postpartum female rats and non-mothers. In the first chapter, I found postpartum female rats to be less anxious than diestrous virgin rats in a light-dark box, a fairly under-utilized behavioral paradigm for studying reproductive state differences in anxiety. The results were consistent with previous work demonstrating reduced anxiety in postpartum rats compared to virgin females in other paradigms (*e.g.,* Fleming & Luebke, 1981; Lonstein, 2007; Toufexis, 2007; Toufexis et al., 1999). In addition, I also confirmed previous reports that postpartum females require recent contact with their litters to display reduced anxiety behavior (Lonstein, 2005; Smith & Lonstein, 2008; Neumann, 2003). Finally, I demonstrated that inhibiting the $GABA_A$ receptor systemically through the GABA, benzodiazepine, and picrotoxin sites has anxiogenic effects in the light-dark box, with inhibition through the picrotoxin site specifically and strongly anxiogenic in dams while often not affecting diestrous virgins. This finding indicates a strong role for the $GABA_A$ receptor picrotoxin binding site in the postpartum reduction of anxiety behavior.

In my second chapter, I used autoradiography to investigate whether there could be differences in the density of binding sites on the $GABA_A$ receptor that could play a role in reproductive state and sex differences in anxiety. Specifically, I expected that postpartum female

rats would have either lower or higher binding to the $GABA_A$ receptor picrotoxin site compared to diestrous virgins, mid-pregnant, and virgin males, as examined using the radioligand, [^{35}S]TBPS. Such a difference could account for postpartum females' different and anxiogenic response in comparison to diestrous virgins to the picrotoxin site agonist, PTZ, in the light-dark box (Chapter 1), and for previously known lower anxiety behavior in postpartum female rats versus mid-pregnant and male virgin rats (Fleming & Luebke, 1981; Neumann et al., 1998; De Brito Faturi et al., 2006; Toufexis, 2007; Toufexis et al., 1999). Results from Chapter 2 instead revealed that dams and virgins did not differ in picrotoxin site binding in any site examined, and that postpartum females did not differ from pregnant or male rats, however, these results must be considered with caution because the [^{35}S]TBPS binding could not be verified as specific. Another surprising result was that diestrous virgins had higher [^{3}H]FNP binding in the hippocampus compared to virgin male and pregnant rats, while postpartum females were not significantly different from any other group in [^{3}H]FNP binding. This difference in [^{3}H]FNP could be related to differential strategies to cope with stressors (Farabonelli et al., 1996) but does not seem to easily relate to group differences in anxiety behavior. [^{3}H]FNP was not different across groups in any other brain region. [^{3}H]MUSC did not differentially bind in any brain region across groups.

In my third chapter, I examined the content of three of the six alpha subunit subtypes of the $GABA_A$ receptor to see whether differences in the composition of the receptor could contribute to reproductive state and sex differences in anxiety behavior. The picrotoxin binding site is associated with the alpha subunit of the $GABA_A$ receptor and binds within the channel pore, as

opposed to benzodiazepines and GABA itself, which bind outside the pore at the α-γ and α-β interfaces of the $GABA_A$ receptor respectively (Burt, 2003; Da Settimo et al., 2007; Smith & Olsen, 1995). As of yet, there is not a consensus as to which alpha subunits might be the most relevant to picrotoxin site binding affinity (Bell-Horner et al., 2000; Fradley et al., 2007; Huang et al., 2001). I used Western blot analysis to determine the expression of the α2, α3, and α4 subunits of the $GABA_A$ receptor as they are all associated with anxiety behavior (Atack et al., 2006; Gulinello et al., 2003; Löw et al., 2000; Smith et al., 1998). I hypothesized that the α2, α3, and α4 subunits would be more highly expressed in regions where GABA activity influences anxiety behavior in postpartum females' brains compared to the other groups because if these subunits do indeed confer high affinity for ligands to the picrotoxin site, having higher expression of these subunits could account for the increased responsiveness to PTZ I found in Chapter 1. In addition, if postpartum females do have much lower concentrations of endogenous picrotoxin agonists compared to more anxious groups, as I hypothesized above, having a higher affinity to the picrotoxin binding site would still be consistent with their lower anxiety. However, the only significant difference in Chapter 3 was that males had higher expression of the α2 subunit in the rPAG than did either postpartum females or diestrous virgins. It is important to note again here that because labeling from the three antibodies used in Chapter 3 was not blocked during my preabsorption controls, that these results must be considered very cautiously and could have been a consequence of non-specific binding. Further work will soon be conducted to reattempt the preabsorption controls necessary to help confirm these data. If my results are reliable, this difference in α2 content could indicate either a sex difference in how the GABAergic system regulates anxiety behavior in the rPAG, possibly through allowing female brains to be ready to respond to neurosteroid fluctuations by downregulating α2 subunits in

preference for neurosteroid sensitive subunits, or that this difference could be related to the rPAG's regulation of behaviors other than anxiety.

It has been known for decades that upregulation in the GABAergic system is involved in reducing anxiety in postpartum female rats (Hansen et al., 1985; Hard & Hansen, 1985), and although there has been some progress in determining how the GABAergic system is involved, there's still a great deal that is unknown. My dissertation work takes a small step towards parsing out these mechanisms. We know that when postpartum females are allowed contact with their pups, their anxiety is decreased and their cerebrospinal fluid GABA content increases (Qureshi et al., 1987), which very likely contributes to postpartum females' reduction in anxiety. One possibility not mentioned earlier is that the really crucial difference in postpartum females could be that they undergo changes throughout pregnancy that set their neural and sensory systems up so that they are attracted to pups and hover over them allowing stimulation of their ventrums, which most female and male virgin rats are very unlikely to do (Fleming & Luebke, 1981; Rosenblatt, 1967). It is possible that the increased attraction to pups, and the resultant increased GABA, are enough to reduce postpartum females' anxiety. However, there could be other changes in the brains of postpartum females' GABAergic system to go along with this increase in GABA content, and there is a need to examine those other possible differences and determine what they could be and how they contribute to the postpartum reduction in anxiety.

In general, my dissertation has provided an elimination of a number of different aspects of the GABAergic system that could have and were among the most likely possibilities to contribute to reproductive state differences in anxiety. A simple total binding difference with more $GABA_A$ receptors available in regions regulating anxiety, or fewer picrotoxin binding sites, could have been a good way for postpartum females to have naturally lower anxiety. In

addition, a change in subunit expression would also have been a relatively simple way for postpartum brains to drive a difference in emotional behavior, especially because we know that changes in either the composition of available $GABA_A$ receptors or synthesis of new $GABA_A$ receptors with different subunit compositions happens frequently in the brain (Arancibia-Carcamo & Moss, 2006; Chen & Olsen, 2007; Kang et al., 2006; Michels & Moss, 2007). Moreover, although I did not find many anxiogenic effects of inhibiting the $GABA_A$ receptor through GABA's own site or the benzodiazepine site, and not any effects specific to postpartum anxiety behavior, this does not necessarily indicate that these binding sites are not important in regulating anxiety behavior in males, non-postpartum females, and even postpartum and diestrous females in other behavioral tests (Atack, 2006; Miller, et al., 2010; Nelovkov et al., 2006). A further implication of my dissertation is that exploratory anxiety paradigms are not always consistent and do not necessarily test identical aspects of anxiety, an idea that has been previously suggested (Bourin & Hascoët, 2003), as my Chapter 1 results for antagonism through the GABA and benzodiazepine sites are not consistent with some previous research in other paradigms.

Identifying what is different in the brains of postpartum females that leads to their reduced anxiety would be a crucial step toward eventually understanding what might be going wrong in individuals with high anxiety. This information is also relevant to a number of other behaviors, as the GABAergic system is involved in many diverse behaviors in addition to anxiety, including sex behavior, maternal behavior, aggression, thermoregulation, food intake, pain sensation, and movement (Arrati et al., 2006; De Almeida et al., 2005; Paredes & Ågmo, 1992). Additionally, because I also examined mid-pregnant and sexually naïve male rats, my

dissertation studies also eliminate some possible mechanisms for sexually dimorphic behaviors and for behavioral differences specific to the mid-pregnancy period.

Future work on this topic could reveal a great deal about GABAergic mechanisms reducing postpartum anxiety behavior. Chapter 3 indicates that it may be unlikely that expression differences in the α2, α3, and α4 subunits in the brain regions examined contribute to anxiety differences in postpartum female rats in comparison to diestrous, mid-pregnant, and virgin male rats, but there are many other $GABA_A$ receptor subunits that remain uninvestigated. For example, the α1 subunit is sometimes indicated in anxiety behavior (Burt, 2003; Korpi & Sinkkonen 2006; Rudolph & Möhler) and is the most highly expressed α subunit in the fore- and midbrain (Da Settimo et al., 2007; Rudolph & Möhler, 2006). The γ2 subunit is strongly indicated in anxiety behavior, and while it is associated with the benzodiazepine binding site (Burt, 2003; Da Settimo et al., 2007; Smith & Olsen, 1995), as mentioned previously the benzodiazepine site is likely involved in postpartum rat anxiety behavior in other exploratory paradigms than the light-dark box (Miller et al., 2010), so this subunit's expression could be informative about GABAergic mechanisms reducing postpartum anxiety, even though it would not necessarily reveal anything about the picrotoxin binding site's involvement. A last, but very important future project is to determine where in the brain infant contact could be eliciting increased GABA release in the brains of postpartum females. We know that postpartum females that are allowed contact with their pups have higher cerebrospinal fluid concentrations of GABA than females separated from their pups (Qureshi et al., 1987) and that separation from pups disrupts postpartum females' reduced anxiety behavior (Lonstein, 2005; Chapter 1), but the neural regions where GABA release is increased have not yet been identified, and this could

reveal a large piece of the puzzle of how and where the GABAergic system contributes to reduced postpartum anxiety.

My dissertation work also has broader implications to other species in addition to rats, including human women. Postpartum women generally experience an increase in positive mood and a decrease in anxiety and other negative emotions during this time period, similar to postpartum female rodents (Altshuler et al., 2000; Breitkopf et al., 2006; Crowley & Roy-Byrnes, 1989; Engle et al., 1990). However, there is a substantial group (5-15%) of postpartum women who experience severe emotional disruption and develop anxiety disorders instead (Altshuler et al., 2000; Engle et al., 1990; Kumar & Robson, 1984). While rodents are obviously not directly comparable to humans in many ways, there are similarities in the GABAergic system's regulation of anxiety across species (Roy-Byrne, 2005; Vermetten & Bremner, 2002). Furthermore, identifying portions of the anxiety neural circuitry that might be different in other animals with high anxiety could eventually influence research in humans and might, at some point, lead us to better treatments for anxiety disorders in postpartum women.

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