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Autoradiographic analysis of GABA_A receptor binding in the neural anxiety network of postpartum and non-postpartum laboratory rats

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Abstract

Postpartum female rats exhibit a suppression of anxiety-related behaviors when compared to diestrous virgin females, pregnant females, and males. This blunted anxiety promotes optimal maternal care and involves elevated GABA neurotransmission, possibly including greater density of GABA_A and benzodiazepine receptors in the postpartum brain. We here examined autoradiographic binding of [³H]muscimol to measure the total population of GABA_A receptors and [³H]flunitrazepam to assess density of benzodiazepine sites in the medial prefrontal cortex, bed nucleus of the stria terminalis, amygdala, hippocampus, and periaqueductal gray of female rats sacrificed on day 7 postpartum, day 10 of pregnancy, or as diestrous virgins. A group of sexually naïve male rats was also included. We found that [³H]muscimol binding did not differ among groups in any site but that diestrous virgin females had greater [³H]flunitrazepam binding in the CA1 and dentate gyrus of the hippocampus compared to mid-pregnant females and males. Notably, postpartum and diestrous virgin females did not significantly differ in binding of either ligand in any site examined. This is the first study to evaluate the densities of GABA_A and benzodiazepine binding sites simultaneously across three female reproductive states and sex with a focus on brain sites influencing anxiety-related behaviors. The results suggest that changes other GABA_A receptor characteristics, such as subunit composition or increased presynaptic GABA release during interactions with offspring, must instead play a greater role in the postpartum suppression of anxiety in laboratory rats.

Keywords

anxiety; autoradiography; fear; GABA; postpartum; sex difference

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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1. Introduction

Motherhood in laboratory rats, mice, and humans is often associated with diminished emotional reactivity to environmental threats [1–3]. In laboratory rodents, this blunted emotional reactivity involves reduced behavioral indices of anxiety and fear, and has important implications for mothers' ability to focus attention on their offspring, fight intruders to the nest, and optimize foraging to support the metabolic demands of lactation [4–9].

Consistent with research on non-parturient mammals [10,11], dampened emotional reactivity in postpartum females involves increased inhibitory neurotransmission at the GABA_A receptor. For example, GABA_A receptor antagonists increase dams' anxiety and fear behaviors to levels found in virgin females, while virgin females treated with GABA_A receptor agonists show low emotional reactivity similar to that of dams [12–15]. Previous studies have shown that lower emotional reactivity in male rats and mice is associated with a greater density of GABA and benzodiazepine binding sites in the cortex, hippocampus, and amygdala [16–22]. Furthermore, in female rats, experimental or endogenous changes in ovarian hormones that affect anxiety behaviors [23,24] alter GABA and benzodiazepine binding in these brain areas [25–30, however see 31].

In the present studies, we hypothesized that reproductive state and sex affect GABA_A receptor density in the neural anxiety network in a manner that could contribute to the postpartum suppression of anxiety-related behaviors. To test this, autoradiography was used to examine binding of [³H]muscimol to the GABA site and of [³H]flunitrazepam to the benzodiazepine site in the medial prefrontal cortex, bed nucleus of the stria terminalis, amygdala, hippocampus, and periaqueductal gray of laboratory rats. We compared females sacrificed on postpartum day 7, pregnancy day 10, on a day of diestrus, and also included a group of sexually naïve male rats. We predicted greater [³H]muscimol and [³H]flunitrazepam binding in the postpartum female brain compared to the other groups, consistent with dams' lower anxiety-related behaviors.

2. Materials and Methods

2.1 Subjects

Subjects were adult female and male Long-Evans rats born and raised in our laboratory, descended from rats purchased from Harlan Laboratories (Indianapolis, IN). After weaning at 21 days old, subjects were housed in groups of 3–4 same-sex littermates in clear polypropylene cages (48 × 28 × 16 cm) with wood shavings for bedding, food and water freely available, and a 12:12 h light/dark cycle. Beginning at day 70 of age, estrous cycles of some females were monitored daily with a vaginal impedance meter (Fine Science Tools, Foster City, CA) and on a day of proestrus, these females were placed overnight in the cage of a sexually experienced Long-Evans male rat in our colony. These females were then housed with 2–3 other mated females. Some of these mated females were placed into a mid-pregnant group ($n = 8$), were housed singly beginning 7 days after mating and sacrificed on day 10 (± 1 day) of pregnancy. The other mated females were allowed to eventually give birth ($n = 8$), and members of this future postpartum group were individually housed 5–6 days before parturition. Their litters were culled to contain 8 pups (4 females and 4 males) within 48 hr after parturition and the dams were sacrificed on day 7 (± 1 day) postpartum. Subjects in the virgin female group ($n = 8$) were singly housed after 70 days of age, vaginally smeared daily and the cytology examined under a microscope to determine stage of the estrous cycle, and rats were sacrificed on a day of diestrus. A group of singly housed, sexually naïve adult males was also included ($n = 8$). Matings were scheduled so that some subjects from each group were always sacrificed concurrently. Sections from two of the

males and two of the pregnant females were inadvertently omitted from the [^3H]flunitrazepam autoradiography, resulting in these groups having a sample size of six for the [^3H]flunitrazepam analysis.

2.2 Tissue collection

Subjects were rendered unconscious with CO_2 and rapidly decapitated using a guillotine. Brains were quickly removed, placed on dry ice, and stored at -80°C until further processing. Brains were later coronally sectioned with a cryostat into six series of $15\text{-}\mu\text{m}$ sections thaw-mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and stored at -80°C until further processing.

2.3 Autoradiography

Two series of slides were thawed at room temperature and the sections were fixed in 1% paraformaldehyde for 2 min. They were then preincubated twice in 50 mM Tris-HCl buffer (pH 7.4) at 4°C for 15 min each. One series of sections was then incubated in 50 mM Tris-HCl buffer containing 10 nM [^3H]muscimol (20–40 Ci/mmol; PerkinElmer, Waltham, MA) [32,33], and the other series was incubated in 50 mM Tris-HCl buffer containing 2 nM [^3H]flunitrazepam (70–87 Ci/mmol; Perkin-Elmer, Waltham, MA) [34–36], for 1 hr at 4°C . Nonspecific binding was determined by incubating slides of adjacent sections in similar solutions of [^3H]muscimol or [^3H]flunitrazepam that contained $100\text{ }\mu\text{M}$ GABA or $10\text{ }\mu\text{M}$ flunitrazepam (Sigma-Aldrich, St. Louis, MO), respectively; nonspecific binding was found to be negligible. After incubation, sections were washed twice in 4°C 50 mM Tris-HCl buffer for 30 sec each, dipped in 4°C dH_2O , then laid out to dry overnight at 4°C .

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) and all were exposed to Hyperfilm MP film at room temperature (Amersham; Perkin-Elmer, Waltham, MA). All groups had slides included in each cassette. Sections incubated with [^3H]muscimol were exposed for 14–28 weeks, while those incubated with [^3H]flunitrazepam were exposed for 14 weeks. Durations of exposure were based on test films developed at various time points. Film was developed and fixed using a Kodak X-OMAT 1000A Processor (Kodak Co., Rochester, NY).

Films were analyzed by placing them on a light box (0.35 Amps, 60 Hz; Knox Manufacturing Co., Wooddale, IL) and relevant images captured using a Zeiss microscope and digital camera (Roper Scientific Photometrics, Tucson, AZ). Brain areas were analyzed bilaterally (Figure 1) using Scion Image to determine optical density. Up to three consecutive sections through each brain site were analyzed for each subject. For a given brain site, multiple optical density measurements were taken bilaterally from each section and the totals averaged by the number of measurements. The optical density totals were averaged across the number of sections analyzed to standardize density values across brain sites. Mixed-model ANOVAs were used to analyze binding of each ligand, with group as the between-subjects factor and brain site as the within-subjects (repeated measures) factor. When omnibus ANOVAs revealed significant main effects or interactions associated with group, Fisher's LSD post-hoc tests were performed to compare differences between pairs of means. Statistical significance was indicated by $p < 0.05$.

3. Results

As expected, there were significant differences among brain sites in [^3H]muscimol binding ($F(13, 364) = 39.9, p = 0.0001$). However, there was no significant main effect of reproductive state/sex ($F(3, 364) = 0.516, p = 0.67$) and no significant interaction between

brain site and reproductive state/sex ($F(39, 364) = 1.21, p = 0.19$; Figure 2). Similarly, there was a significant main effect of brain site for [^3H]flunitrazepam binding ($F(13, 312) = 323.71, p = 0.0001$) but no significant main effect of reproductive state/sex ($F(3, 312) = 0.46, p = 0.72$). There was a significant site by group interaction ($F(39, 312) = 1.89, p = 0.0017$), though. Post-hoc analyses revealed that diestrous virgins had significantly greater [^3H]flunitrazepam binding in both the DG and CA1 compared to that found in mid-pregnant or male rats ($ps < 0.05$). Postpartum females did not significantly differ from any other group in [^3H]flunitrazepam binding in the hippocampus or any other brain site (Figure 3).

4. Discussion

Heightened GABA_A receptor activity is known to be responsible, at least in part, for the postpartum reduction of anxiety-related behaviors [12–15]. We hypothesized that differences in neural GABA_A receptor binding could contribute to the reproductive state difference in anxiety-related behaviors. This was partly based on data showing higher GABA and benzodiazepine receptor binding in the cortex, hippocampus, and amygdala of low-anxiety male rats [16–22] and that binding in these brain areas is affected by endocrine status in virgin female rats [25–30]. The GABA and benzodiazepine binding sites were of particular interest because dams' emotional behaviors revert to virgin levels when the GABA binding site is antagonized with bicuculline [15] or when the benzodiazepine site is inversely agonized with FG-7142 [14].

Other studies have compared GABA_A and benzodiazepine receptor binding in postpartum and virgin female rat brains, but because some important questions remained unanswered, we were prompted to reexamine this topic. Majewska and colleagues [37] compared [^3H]muscimol binding in the homogenized forebrains of cycling, pregnant and day 2 postpartum rats and found an increase in binding affinity during late pregnancy. They reported no difference in receptor density, but the very large amount of brain tissue included in the homogenates could have obscured subregional differences among groups. Ferreira et al. [12] compared day 5–8 postpartum and cycling virgin females and found no differences in [^3H]muscimol binding in homogenates of the cortex and hippocampus and [^3H]flunitrazepam binding in the cortex, hippocampus, amygdala, and hypothalamus. Concas et al. [26] also reported no difference between estrous virgins and day 7 postpartum rats in [^3H]GABA or [^3H]flunitrazepam binding in the homogenized cortex. Some possible advantages of the present study included the use of radioligand-labeled brain sections on slides exposed to film to greatly increase neuroanatomical localization, examining a larger number of brain sites where GABA_A receptor ligands have been previously observed to modulate anxiety, and controlling for virgin females' stage of the estrous cycle because ovarian and pituitary hormones tremendously affect anxiety-related behavior [23,24]. Even so, we revealed no differences between postpartum and diestrous rats in the density of GABA_A and benzodiazepine receptor binding in the brain regions examined. Thus, based on our results and those of other laboratories, it seems reasonable to conclude that differences in density of these receptors within the brain areas examined cannot be responsible for differences between postpartum and non-postpartum rats in their anxiety-related behaviors.

We did find significant differences in ligand binding among brain sites, with the highest [^3H]muscimol binding found in the CA1 and DG of the hippocampus whereas the highest [^3H]flunitrazepam binding was in the mPFC and DG. Some similar findings can be found in the literature on male rats [18, 38–40]. The inclusion of both male and female virgins in our study also allowed us to also reveal a sex difference, with greater [^3H]flunitrazepam binding in the CA1 and DG of the hippocampus of diestrous virgins compared to that found in males. This sex difference in hippocampal [^3H]flunitrazepam binding could be associated with sex differences in the emotional behaviors, HPA axis responses to stress, or the

cognitive functions relying on this structure. Earlier studies examining sex differences in hippocampal GABA_A receptor density have been inconsistent. Shephard et al. [41] reported that males had a higher concentration of hippocampal [³H]flunitrazepam binding than did females, while Wilson [42] reported no sex difference in hippocampal [³H]flunitrazepam binding. Comparing our results to those of Shephard et al. [41] is particularly difficult because their rats experienced food deprivation, avoidance conditioning, and neophobia testing before sacrifice. We found no sex difference in [³H]flunitrazepam or [³H]muscimol binding in the CeA and mPFC, which is consistent with some reports [42–45], but a sex difference (female > male) in benzodiazepine binding in the cortex has also been seen [41,43] as well as a small but significant sex difference (M > F) in [³H]muscimol binding in the cortex [46]. The present study appears to be the first to compare female and male laboratory rats in GABA_A receptor binding in the BST and PAG. Although we found no sex difference in either site, this could be species-dependent because female wild wood mice (*Apodemus sylvaticus*) have considerably higher [³H]muscimol binding in both sites compared to that found in males [47].

The similar levels of GABA and benzodiazepine receptor binding in postpartum and diestrous rats reported here and by others [12,37] will redirect our focus on other characteristics of central GABA systems that could be influenced by maternal state and contact with infants. For example, the GABA_A receptor has other ligand binding sites not very often implicated in anxiety, but could be worth examining. In fact, we have data indicating that pentylenetetrazol (PTZ), a picrotoxin site agonist that blocks the chloride ion channel, is tremendously anxiogenic in postpartum but not virgin female rats tested in a light-dark box [Miller, Piasecki and Lonstein, in preparation]. The neural anxiety networks may also differ between dams and virgins in expression of the subunit proteins comprising the GABA_A receptor. The receptor's subunit composition greatly affects its ligand-binding affinity and pharmacology, as well as anxiety behavior [48,49]. Even so, while cortical and hippocampal expression of some α , β and γ subunits change across late pregnancy and the early postpartum period, most return to pre-mating baseline by postpartum day 7 [26,50], which is when anxiety differs most between dams and virgins [7]. Two exceptions are the $\alpha 4$ and δ subunits, which show elevated mRNA and lower immunoreactivity in the hippocampus on day 7 postpartum, respectively [26,51]. These particular subunits mediate the anxiety-modulating effects of neuroactive steroids, such as those fluctuating during the peripartum period [52–55], but their role is also questionable for the suppression of anxiety behavior thereafter because neuroactive steroids levels are low again by postpartum day 2 [56] and dams' elevated plus-maze behavior is unaffected by steroidogenesis inhibition [57, Cavanaugh and Lonstein, unpublished data].

In addition to any postsynaptic factors, functionally relevant differences between virgin and day 7 postpartum female rats in central GABA systems surely exist *presynaptically*. Cerebral spinal fluid GABA concentrations are high in day 5–7 postpartum female rats when dams are allowed to physically interact with pups [58] and this may also be true for the production and release of endogenous benzodiazepines or other positive modulators of the GABA_A receptor [59–61]. Dams may also have more efficient vesicular packaging and release of these ligands, or reduced ligand re-uptake from synapses in brain sites associated with anxiety. Indeed, virgins and dams do not differ in cortical GABA_A receptor binding or synaptosomal chloride uptake in response to a given amount of GABA or muscimol [56,57], but rat mothers have higher GABA turnover in their cingulate cortex [61], indicating that this could be one plausible presynaptic mechanism for reducing anxiety in postpartum females. Alternatively, dams may have lower production and release of negative receptor modulators, including those targeting the picrotoxin site [62].

In sum, the present studies investigated possible differences in GABA and benzodiazepine site binding among virgin, pregnant and postpartum rats in a large number of brain sites where GABA receptor activity influences anxiety behavior. The results, along with the results of previous studies examining GABA_A ligand binding, strongly suggest that differences in the density of postsynaptic binding sites cannot explain reproductive-state differences in anxiety-related behaviors. Future attention to presynaptic GABA-related mechanisms could reveal them as more important contributors to the blunted anxiety-related behavior of postpartum female rats.

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Abbreviations

aco	anterior commissure
AQ	cerebral aqueduct
BSTd	dorsal bed nucleus of the stria terminalis
BSTl	lateral bed nucleus of the stria terminalis
BSTv	ventral bed nucleus of the stria terminalis
CeA	central nucleus of the amygdala
cPAG	caudal periaqueductal gray
DG	dentate gyrus
fa	fascicle
Hipp	Hippocampus
mPFC	medial prefrontal cortex
opt	optic tract
rPAG	rostral periaqueductal gray

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Research Highlights

- Present study is the first to examine GABA and BDZ autoradiographic binding in the neural anxiety network simultaneously across three female reproductive states and sex in laboratory rats
- BDZ binding in the CA1 and DG of the hippocampus was greater in virgin females compared to pregnant females and males
- Particularly interesting finding that postpartum females did not differ from virgin females in GABA or BDZ binding, suggesting the postpartum suppression of anxiety behavior is associated with other GABA system characteristics.

Figure 1.

Photomicrographs of representative brain sections exposed to [^3H]flunitrazepam. Black boxes indicate areas of optical density analysis in the (A-F): medial prefrontal cortex (PFC), dorsal, ventral and lateral areas of the bed nucleus of the stria terminalis (BST), central amygdala (CeA), hippocampus (Hipp), and rostral and caudal periaqueductal gray (rPAG, cPAG). 3V = third ventricle, aco = anterior commissure, AQ = cerebral aqueduct, fa = fascicle, opt = optic tract.

Figure 2. $[^3\text{H}]$ Muscimol autoradiographic binding (nCi/mg; Mean + SEM) in the brains of postpartum day 7, pregnancy day 10, diestrous virgin, and male virgin rats.

Figure 3.

[³H]Flunitrazepam autoradiographic binding (nCi/mg; Mean + SEM) in the brains of postpartum day 7, pregnancy day 10, diestrous virgin, and male virgin rats. * = diestrous virgins significantly different from pregnant and male rats ($p < 0.05$).