Pharmacology, Biochemistry and Behavior 100 (2011) 130–137

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Use of the light–dark box to compare the anxiety-related behavior of virgin and postpartum female rats

Stephanie M. Miller [a], Christopher C. Piasecki [a,b], Joseph S. Lonstein [a,b,*]

[a] Behavioral Neuroscience Program, Department of Psychology, 108 Giltner Hall, Michigan State University, East Lansing, MI 48824, USA
[b] Neuroscience Program, 108 Giltner Hall, Michigan State University, East Lansing, MI 48824, USA

ARTICLE INFO

Article history:
Received 19 May 2011
Received in revised form 11 July 2011
Accepted 2 August 2011
Available online 7 August 2011

Keywords:
Anxiety
Black–white box
Fear
GABA
Lactation
Maternal behavior

ABSTRACT

Postpartum female rodents are less anxious than diestrous virgins and this difference contributes to dams' ability to adequately care for pups and defend the nest. Low postpartum anxiety has been observed in many behavioral paradigms but the results of previous studies using the light–dark box have been inconsistent. We here reexamined the usefulness of the light–dark box to assess differences between postpartum and diestrous virgin female rats in their anxiety-related behavior. We found a significant effect of reproductive state, such that dams spent more time in the light chamber than did diestrous virgins. This difference required recent physical contact with pups because a four-hour separation from pups reduced dams' time spent in the light chamber by half, similar to what we previously found for litter-separated dams tested in an elevated plus maze. We then examined if dams' low-anxiety behavior in the light–dark box depends on high $GABA_A$ receptor activity by inhibiting the receptor at different binding sites using (+)-Bicuculline to target the GABA site, FG-7142 to target the benzodiazepine site, and pentylenetetrazol to target the picrotoxin site. Only pentylenetetrazol was consistently anxiogenic in dams, while having little effect in diestrous virgins. Thus, the light–dark box can be a useful paradigm to study differences between postpartum and diestrous virgin female rats in their anxiety-related behaviors, and this difference is influenced by dams' recent contact with pups and $GABA_A$ receptor neurotransmission particularly affected by activity at the picrotoxin site.

1. Introduction

Female rodents undergo complex behavioral changes during the peripartum period, including decreased fear- and anxiety-related behaviors that may be prerequisite for dams' heightened maternal responsiveness to neonates and aggression toward intruders (Fleming and Luebke, 1981; Hard and Hansen, 1985). Recently parturient females exhibit less anxiety behavior in many paradigms when compared to pregnant or virgin females (for reviews see Lonstein, 2007; Neumann, 2003), but the light–dark box has been surprisingly under-utilized to study postpartum anxiety given its simplicity and frequent use for studying anxiety in male rodents. 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 and D'Amato, 1991), but a more recent study suggests no difference in light–dark box behavior between postpartum and virgin mice (Gammie et al., 2008). One study of female rats also found no significant difference between postpartum and diestrous virgins in light–dark box behavior (Zuluaga et al., 2005).

Converging results among multiple behavioral paradigms would help increase assurance about the most relevant influences on postpartum anxiety, as opposed to often indefinable aspects of anxiety idiosyncratically revealed by a single paradigm (Ramos, 2008). The usefulness of such convergence, in addition to the wealth of evidence demonstrating low postpartum anxiety in most behavioral paradigms (for some exceptions see Lonstein, 2007) and discrepancies among the existing studies using light–dark boxes to compare anxiety across female reproductive states, we here re-examined the effect of motherhood on light–dark box behaviors in female rats. In a second experiment, we tested if recent contact with pups is required for dams' reduced anxiety-related behavior in a light–dark box, as we have found in an elevated plus-maze (Figueira et al., 2008; Lonstein, 2005; Smith and Lonstein, 2008). In a final experiment, we investigated the influence of $GABA_A$ receptor activity on light–dark box behavior in both postpartum and virgin female rats. Similar to anxiety in non-postpartum animals (Millan, 2003), elevated postpartum $GABA_A$ neurotransmission is strongly implicated in dams' low anxiety behavior in other paradigms (Hansen, 1990; Hansen et al., 1985; Miller et al., 2010). To pinpoint which $GABA_A$ receptor binding sites may be particularly involved in suppressing dams' anxiety behavior in the light–dark box, in separate groups of subjects we targeted the GABA site with (+)-Bicuculline [(+)-Bic], the benzodiazepine site with FG-7142, and the picrotoxin site with pentylenetetrazol [PTZ].

* Corresponding author. Tel.: +1 517 353 8675; fax: +1 517 432 2744.
E-mail address: lonstein@msu.edu (J.S. Lonstein).

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doi:10.1016/j.pbb.2011.08.002

2. Experiment I: light–dark box behavior of postpartum and diestrous virgin rats

2.1. Methods

2.1.1. Subjects

Subjects were female Long–Evans rats, descended from rats purchased from Harlan Laboratories (Indianapolis, IN), born and raised in our colony. After weaning at 21 days of age, subjects were group-housed in clear polypropylene cages (48×28×16 cm) in groups of 2–3 female littermates, with wood shavings for bedding, a 12:12 light:dark cycle (0800 h lights on), and food and water available *ad lib*. After 75 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 group 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 before the expected day of parturition, these females were singly housed. Litters were culled to contain 4 males and 4 females within 48 h after birth. All procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee at Michigan State University.

2.1.2. Light–dark box testing

Behavioral testing occurred between 1200 and 1630 h. Dams were tested on day 7 or 8 postpartum ($n=9$) with the day of parturition assigned as day 0. Virgin females ($n=8$) were gently vaginally smeared each morning and the cytology used to determine their stage of the estrous cycle; females were tested on a day of diestrus. Subjects were left undisturbed in the colony room at least 3 h before testing, then brought in a cage to a nearby testing room containing a light–dark box. The light–dark box was made of white and black opaque Plexiglas (20×30×30 cm light chamber, 30×30×30 cm dark chamber). The chambers were connected by a 10×10 cm door in the middle of the wall separating the two chambers. Animals were placed in the middle of the light chamber facing a side away from the door and then released.

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 scored for 10 min with a computerized data acquisition system either while being videotaped, or the videotapes were transcribed at a later time. Ambient illumination was 624 lx in the light chamber and 3 lx the dark chamber because our previous pilot work revealed that light levels lower than this resulted in no difference between postpartum and diestrous virgin female rats in their light–dark box behavior (Miller and Lonstein, 2006). After testing, subjects were removed from the light–dark box and returned to their home cage in colony room. The apparatus was cleaned with 70% ethanol after each use and allowed to dry before the next subject was tested.

2.1.3. Behavioral variables

Behaviors in the light–dark box 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 (at least part of the head but not all 4 feet in 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 spent in the dark chamber (non-responders were assigned a latency of 600 s). These behaviors have all previously been measured as a reflection of anxiety in this apparatus (Costall et al. 1989; Crawley, 1981; Bourin and Hascöet, 2003; De Angelis 1992; Hascöet and Bourin, 1998). Because the frequency of rears in the light chamber is easily confounded by the duration of time animals spend in the light chamber, the frequency of rears made in the light chamber was standardized by the duration of time each subject spent in that chamber (number of rears/duration of time in light×100).

2.1.4. Data analyses

Data were first examined for normality using Shapiro–Wilk's tests. Normally distributed data were analyzed with independent *t*-tests comparing postpartum and virgin rats. Variables not normally distributed were log transformed and normality confirmed on the transformed data prior to analyses with *t*-tests. In all cases, statistical significance was indicated by $p<0.05$.

2.2. Results

Postpartum females spent significantly more time in the light chamber of the light–dark box than did diestrous virgins ($t(15)=2.21$, $p<0.05$; Fig. 1). Dams also tended to transition between chambers significantly more often than did virgins (Table 1). The frequency of rears standardized by the duration of time subjects spent in the light did not differ between groups. Groups also did not differ in the frequency of stretches from the dark chamber to the light chamber, their latencies to initially enter the dark chamber, or in the other behavioral variables recorded (Table 1).

3. Experiment II: influence of recent litter contact on light–dark box behavior of postpartum rats

3.1. Methods

3.1.1. Subjects

Subjects were postpartum female rats from our colony raised and housed as described in Experiment I.

3.1.2. Light–dark box testing

Testing followed the same procedure as Experiment I, except that one group of postpartum females ($n=15$) had their pups removed and placed in an incubator set at 34 °C (nest temperature) 4 h before testing. We previously found this increases postpartum female rats' anxiety-related behavior in an elevated plus-maze (Figueira et al., 2008; Lonstein, 2005; Smith and Lonstein, 2008). The other group of dams ($n=16$) were left alone in their home cages and allowed

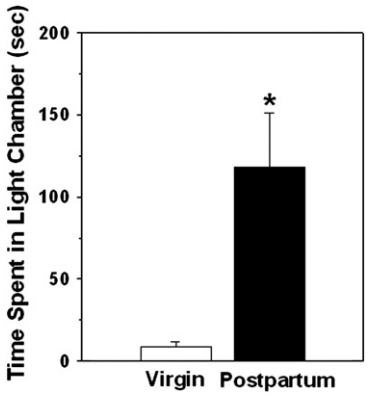

Fig. 1. Duration of time spent in the light chamber of a light–dark box by diestrous virgin (white bar) and postpartum (black bar) rats. Data were not normally distributed so log-transformed prior to analysis. Untransformed means ± SEMs are shown. $^{*}p<0.05$ on the transformed data.

Table 1
Anxiety-related behaviors of unmanipulated diestrous virgin and postpartum female rats tested in a light–dark box. *Includes responders only. #Not normally distributed so log-transformed prior to analysis. Non-transformed means ± SEM are shown in all cases. Statistical significance was indicated by $p<0.05$.

	Virgin	Postpartum	$t(15)$	p
Frequency of chamber transitions#	3 ± 1	10 ± 2	1.93	0.07
Frequency of rears/time spent in light chamber*#	14 ± 8	14 ± 2	0.49	0.63
Frequency of stretches from dark to light	24 ± 3	27 ± 3	0.57	0.58
Latency to enter dark chamber (s)#	2 ± 1	5 ± 3	0.21	0.83
Latency to re-enter light chamber (s)#	314 ± 107	178 ± 82	0.81	0.43

Table 2
Anxiety-related behaviors (mean ± SEM) of postpartum rats separated from pups or not 4 h before testing in a light–dark box. *Includes responders only. #Not normally distributed so log-transformed prior to analysis. Non-transformed means ± SEM are shown in all cases. +One-tailed tests; statistical significance was indicated by $p<0.05$.

	Unseparated	Separated	$t(29)$	p^+
Frequency of chamber transitions	10 ± 2	7 ± 1	1.78	0.04
Frequency of rears/time spent in light chamber*	15 ± 2	13 ± 2	0.60	0.28
Frequency of stretches from dark to light	24 ± 2	24 ± 3	0.04	0.48
Latency to enter dark chamber (s)#	6 ± 5	1 ± 0	0.60	0.28
Latency to re-enter light chamber (s)#	155 ± 56	140 ± 52	0.02	0.49

continual contact with their pups until the time of testing. Separated litters were returned to their dams immediately after testing.

3.1.3. Data analyses

Based on our previous results demonstrating that separation from pups significantly increases dams' anxiety-related behaviors (Figueira et al., 2008; Lonstein, 2005; Smith and Lonstein, 2008), data from this experiment were analyzed using one-tailed *t*-tests. Variables that were not normally distributed were log transformed before analysis. Statistical significance was indicated by $p<0.05$.

3.2. Results

Dams that were separated from their pups 4 h before testing spent approximately half as much time in the light chamber compared to unseparated dams ($t(29)=1.79$ $p<0.05$; Fig. 2). Separated dams also exhibited significantly fewer chamber transitions than did dams allowed continual access to their pups before testing (Table 2). The groups did not differ in the other behavioral variables recorded (Table 2).

4. Experiment III: effects of $GABA_A$ receptor antagonism on light–dark box behavior of postpartum and diestrous virgin rats

4.1. Methods

4.1.1. Subjects

Subjects were housed as described in Experiment I, with the exception that virgins in this experiment were singly housed at least 3 days before testing. We previously found no effect of single *vs.* group housing on diestrous virgin females' behavior in an elevated plus-maze; in both cases virgins display more anxiety-related behaviors than do postpartum females (Lonstein, unpublished data).

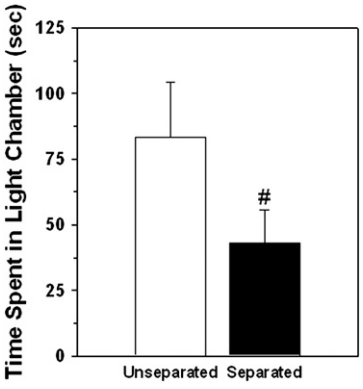

Fig. 2. Duration of time (mean ± SEM) spent in the light chamber of a light–dark box by postpartum rats that were either allowed access to their litters (Unseparated) or separated from their litters 4 h before testing (Separated). Data were not normally distributed so log-transformed prior to analysis. Untransformed means ± SEMs are shown. #$p<0.05$, one-tailed on the transformed data.

4.1.2. Drugs

All drugs were purchased from Sigma (USA). (+)-Bicuculline [(+)-Bic] (2 or 4 mg/kg) was prepared similar to that described by 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 5.0, and then the volume raised to 1 mL. This provided 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) was dissolved in physiological saline. Doses used were based on those previously observed to affect anxiety-related behaviors in laboratory rodents (Atack et al., 2005; Evans and Lowry, 2007; File, 1984; Hansen et al., 1985; Nicolas and Prinssen, 2006; Pellow and File, 1986; Dalvi and Rodgers, 1996; Zarrindast et al., 2001). The injected volume of all solutions was 1 mL/kg body weight. Control animals for each drug received the corresponding vehicle in which the drug was dissolved. To avoid additional handling and stress on the day of testing, subjects' body weights were obtained 1–4 days before testing.

4.1.3. Light–dark box testing

Light–dark box testing was conducted similar to Experiment I above. Subjects were briefly removed from their home cage within the colony room, received an intraperitoneal injection of vehicle or one of the three drugs, returned to their home cage, and brought to the nearby testing room 15 min later; this time of testing after injection was used for all three drugs to facilitate comparison across the them and is consistent with the window of action for these drugs after intraperitoneal injection (*e.g.*, Cole et al., 1995; Miller et al., 2010; Uehara et al., 2005). There were 11–16 animals in each group (see Tables 3–5 for sample sizes).

4.1.4. Data analyses

Data were analyzed with a series of separate 2 (reproductive state) × 3 (drug dose) ANOVAs for each of the drugs tested. Significant omnibus ANOVAs were followed by Fisher's LSD post-hoc tests comparing individual groups. Data that were not normally distributed according to Shapiro–Wilk's tests were log transformed prior to analyses with the ANOVAs. In cases of significant interactions, one-way ANOVAs were used to compare groups within reproductive state. 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 ($p<0.01$).

Table 3
Anxiety-related behaviors (mean ± SEM) of diestrous virgin and postpartum female rats tested in a light–dark box after IP injection of vehicle or (+)-Bic. *Includes responders only. #Not normally distributed so log-transformed prior to analysis. Non-transformed means ± SEM are shown in all cases. Statistical significance was indicated by $p<0.05$. See text for additional statistical details.

	Virgin			Postpartum			Significant effects
	Vehicle ($n=16$)	(+)-Bic 2 mg ($n=12$)	(+)-Bic 4 mg ($n=12$)	Vehicle ($n=13$)	(+)-Bic 2 mg ($n=15$)	(+)-Bic 4 mg ($n=12$)	
Frequency of chamber transitions#	4 ± 1	2 ± 0	6 ± 2	11 ± 1	7 ± 1	7 ± 1	State, Dose
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 (s)#	6 ± 3	3 ± 2	7 ± 4	0 ± 0	2 ± 2	2 ± 1	State
Latency to re-enter the light chamber (s)#	418 ± 60	466 ± 69	245 ± 81	86 ± 45	228 ± 71	196 ± 71	State, State × Dose

4.2. Results

4.2.1. (+)-Bicuculline

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 did virgins ($F(1,74)=10.10$, $p<0.002$; Fig. 3). There was no significant main effect of (+)-Bic ($F(2,74)=2.51$, $p>0.09$), or interaction between reproductive state and (+)-Bic ($F(2,74)=1.33$, $p>0.27$), on the duration of time spent in the light chamber.

There was also a significant main effect of reproductive state on the frequency of chamber transitions such that dams transitioned more often than virgins ($F(1,74)=18.29$, $p<0.0001$; Table 3). There was also a main effect of (+)-Bic on chamber transitions ($F(2,74)=3.18$, $p<0.05$) and post-hoc analysis revealed that vehicle-injected subjects transitioned more often than subjects receiving 2 mg of (+)-Bic. There was no significant interaction between reproductive state and (+)-Bic on the number of chamber transitions ($F(2,74)=2.18$, $p>0.12$).

There was a significant main effect of reproductive state on the frequency of rears standardized for the duration of time subjects spent in the light chamber, with dams rearing more often than did virgins ($F(1,72)=4.83$, $p<0.04$). There was no significant effect of (+)-Bic ($F(2,72)=1.52$, $p>0.22$) (Table 3) or interaction between these factors ($F(2,72)=2.01$, $p>0.13$) on this rearing measure.

There were also no main effects of reproductive state ($F(1,74)=0.10$, $p>0.75$) or (+)-Bic ($F(2,74)=2.16$, $p>0.12$) on the frequency of stretches from the dark chamber to the light chamber (Table 3). There was also no significant interaction between these factors ($F(2,74)=0.91$, $p>0.41$) on the frequency of stretches.

The latency to re-enter the light chamber after spending time in the dark chamber was significantly shorter in dams than in virgins ($F(1,74)=13.76$, $p<0.0005$; Table 3) but there was no main effect of (+)-Bic ($F(2,74)=1.67$, $p>0.19$). There was a significant interaction between reproductive state and (+)-Bic on this measure ($F(2,74)=4.42$, $p<0.02$), but analysis within reproductive state revealed that (+)-Bic only marginally reduced the latency in virgins at the 4 mg/kg dose (one way ANOVA — $F(2,37)=2.54$, $p=0.09$) and had no effect in dams (one way ANOVA — $F(2,37)=1.40$, $p>0.25$).

4.2.2. FG-7142

There was a main effect of reproductive state ($F(1,80)=10.23$, $p<0.003$), but not FG-7142 ($F(2,80)=0.08$, $p>0.92$) and no interaction between these factors ($F(2,80)=1.09$, $p>0.34$), on the duration of time females spent in the light chamber (Fig. 4).

The frequency of chamber transitions was significantly higher in dams than in virgins ($F(1,80)=19.20$, $p<0.0001$; Table 4). There was no significant main effect of FG-7142 ($F(2,80)=1.03$, $p>0.36$) and no significant interaction between reproductive state and FG-7142 on the number of transitions ($F(2,80)=1.12$, $p>0.37$).

There were no significant main effects of reproductive state ($F(1,80)=2.35$, $p>0.12$) or FG-7142 ($F(2,80)=0.27$, $p>0.76$), and no interaction between these factors ($F(2,80)=1.58$, $p>0.21$), on the frequency of rears standardized for the amount of time subjects spent in the light chamber (Table 4).

There were no significant main effect of reproductive state ($F(1,80)=1.92$, $p>0.16$) on the frequency of stretches made from the dark chamber to the light chamber. There was a significant effect of FG-7142, though ($F(2,80)=7.01$, $p<0.002$), with the lowest dose reducing them compared to vehicle. There was no significant interaction between these factors ($F(2,80)=0.46$, $p>0.63$) on these stretches (Table 4).

The latency to re-enter the light chamber after the first bout of time spent in the dark chamber was shorter in dams than in virgins ($F(1,80)=10.97$, $p<0.002$), but this was not affected by FG-7142 ($F(2,80)=1.52$, $p>0.22$) and there was no significant interaction

Table 4
Anxiety-related behaviors (mean ± SEM) of diestrous virgin and postpartum female rats tested in a light–dark box after IP injection of vehicle or FG-7142. *Includes responders only. #Not normally distributed so log-transformed prior to analysis. Non-transformed means ± SEM are shown in all cases. Statistical significance was indicated by $p<0.05$. See text for additional statistical details.

	Virgin			Postpartum			Significant effects
	Vehicle ($n=12$)	FG-7142 10 mg ($n=16$)	FG-7142 25 mg ($n=17$)	Vehicle ($n=15$)	FG-7142 10 mg ($n=12$)	FG-7142 25 mg ($n=14$)	
Frequency of chamber transitions#	4 ± 1	3 ± 1	4 ± 1	10 ± 1	6 ± 1	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 (s)#	4 ± 2	50 ± 37	11 ± 4	1 ± 0	11 ± 7	7 ± 6	–
Latency to re-enter the light chamber (s)#	431 ± 67	309 ± 75	338 ± 69	103 ± 39	135 ± 64	301 ± 65	State

Table 5
Anxiety-related behaviors (mean ± SEM) of diestrous virgin and postpartum female rats tested in a light–dark box after IP injection of vehicle or PTZ. *Includes responders only. #Not normally distributed so log-transformed prior to analysis. Non-transformed means ± SEM are shown in all cases. Statistical significance was indicated by $p<0.05$. Groups with different superscript letters significantly differ, one-way ANOVA within reproductive state, $p<0.05$. See text for additional statistical details.

	Virgin			Postpartum			Significant effects
	Vehicle ($n=12$)	PTZ 10 mg ($n=10$)	PTZ 20 mg ($n=12$)	Vehicle ($n=14$)	PTZ 10 mg ($n=14$)	PTZ 20 mg ($n=12$)	
Frequency of chamber transitions#	3 ± 1	3 ± 1	3 ± 1	12 ± 2[a]	9 ± 1[a]	2 ± 1[b]	State, Dose, State × Dose
Frequency of rears/time spent in light chamber*	9 ± 4	8 ± 3	3 ± 2	15 ± 1	10 ± 2	6 ± 6	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 (s)#	2 ± 1	4 ± 1	3 ± 2	1 ± 0	2 ± 1	1 ± 1	State
Latency to re-enter the light chamber (s)#	389 ± 76	429 ± 86	459 ± 66	114 ± 46	94 ± 22	492 ± 65	State, Dose

between factors on this latency measure ($F(2,80)=3.11$, $p>0.05$; Table 4).

4.2.3. Pentylenetetrazol

Dams spent significantly more time in the light chamber compared to virgins ($F(1,68)=11.05$, $p<0.002$; Fig. 5). PTZ significantly affected the time females spent in the light chamber, with the 20 mg dose decreasing it compared to 10 mg of PTZ or saline ($F(2,68)=8.05$, $p<0.0008$; Fig. 5). This was qualified by a significant interaction between reproductive state and dose of PTZ ($F(2,68)=8.59$, $p<0.0006$), such that dams receiving the 20 mg dose of PTZ spent less time in the light chamber compared to other groups of dams (one way ANOVA — $F(2,37)=4.98$, $p<0.02$), while PTZ had no effect on the duration of time virgins spent in the light chamber (one way ANOVA — $F(2,31)=0.35$, $p>0.71$) (Fig. 5).

Postpartum females made significantly more chamber transitions than did virgins ($F(1,68)=21.28$, $p<0.0001$) and this was also affected by PTZ such that the 20 mg dose significantly reduced chamber transitions ($F(2,68)=9.46$, $p<0.0003$). Similar to the duration of time spent in the light chamber, there was an interaction between reproductive state and PTZ ($F(2,68)=8.66$, $p<0.0005$), with 20 mg PTZ reducing chamber transitions in dams (one way ANOVA — $F(2,37)=11.66$, $p<0.0001$) but not virgins (one way ANOVA - $F(2,31)=0.03$, $p>0.96$) (Table 5).

Reproductive state did not influence the frequency of rears standardized for the duration of time subjects spent in the light chamber ($F(1,41)=2.08$, $p>0.15$). There was, however, a significant main effect of PTZ with 20 mg significantly reducing rears compared to the 10 mg dose or saline ($F(2,41)=4.71$, $p<0.02$). There was no significant interaction between reproductive state and PTZ on the standardized frequency of rears ($F(2,41)=0.31$, $p>0.73$; Table 5).

The was also no main effect of reproductive state ($F(1,68)=2.34$, $p>0.13$) on the frequency of stretches from the dark chamber to the light chamber. There was, however, a significant main effect of PTZ, with 20 mg significantly decreasing stretches from the dark chamber ($F(2,68)=12.57$, $p<0.0001$). There was no significant interaction between factors on frequency of stretching from the dark chamber ($F(2,68)=2.76$, $p>0.07$; Table 5).

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 dams re-entering the light chamber faster than did virgins ($F(1,68)=10.86$, $p<0.002$). 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 ($F(2,68)=4.80$, $p<0.02$). There was no significant interaction between reproductive state and PTZ on this latency measure ($F(2,68)=2.33$, $p>0.10$) (Table 5).

5. Discussion

The present results demonstrate that: 1) postpartum rats display fewer anxiety-related behaviors in a light–dark box than do diestrous virgins, 2) separating mothers from their pups 4 h before testing increases anxiety-related behaviors in a light-dark box when these separated females are compared to dams not separated from their litter before testing, and 3) (+)-Bic and FG-7142 each produced some minor anxiogenic effects in both dams and diestrous virgins, but the picrotoxin site ligand PTZ was potently and selectively anxiogenic in postpartum females.

5.1. Methodological considerations

Our results demonstrate that postpartum rats display behaviors reflecting low anxiety in a light–dark box — including more time spent in the light chamber, more transitions between chambers, and in

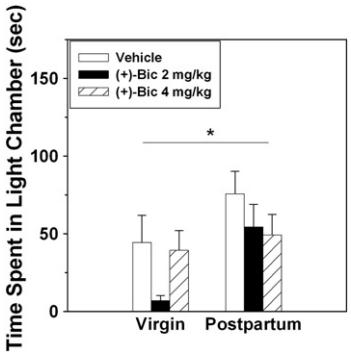

Fig. 3. Duration of time (mean ± SEM) spent in the light chamber by diestrous virgin and postpartum rats that received IP injection of vehicle, 2 mg (+)-Bic, or 4 mg (+)-Bic. Data not normally distributed so log-transformed prior to analysis. Untransformed means ± SEMs are shown. *Significant main effect of reproductive state on the transformed data, $p<0.05$.

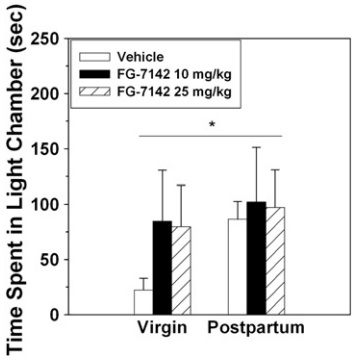

Fig. 4. Duration of time spent in the light chamber by diestrous virgin and postpartum rats that received IP injection of vehicle, 10 mg FG-7142, or 25 mg FG-7142. Data not normally distributed so log-transformed prior to analysis. Untransformed means ± SEMs are shown. *Significant main effect of reproductive state on the transformed data, $p<0.05$.

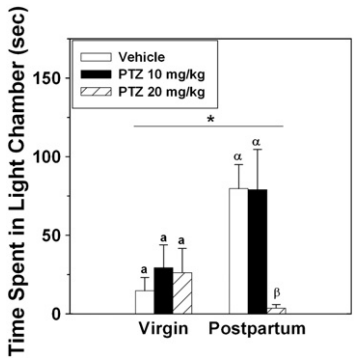

Fig. 5. Duration of time (mean ± SEM) spent in the light chamber by diestrous virgin and postpartum rats that received IP injection of vehicle, 10 mg PTZ, or 20 mg PTZ. *Significant main effect of reproductive state on the transformed data, $p < .05$. Different Roman or Greek letters above bars within each reproductive state indicate significant difference among groups, post-hoc $p < 0.05$.

some of the experiments a greater readiness to reenter the light chamber (Chaouloff et al., 1997; Crawley, 1981; Ramos et al., 1997) — when compared to diestrous virgins. These results are consistent with a great deal of previous research using other behavioral paradigms to compare anxiety across reproductive states in female rats (Lonstein, 2007). However, the results of the three previous studies examining how postpartum state impacts behavior in a light–dark box are inconsistent. Similar to our findings, 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. In contrast, Gammie et al. (2008) found no significant change in the behavior of female mice tested in a light–dark box during pregnancy and through early lactation. Zuluaga et al. (2005) also found no significant differences between postpartum and diestrous virgin rats in their light–dark box behavior. Although our focus here is on the light–dark box, postpartum and virgin female rats have sometimes been reported to also not differ in their behavior in an elevated plus maze (Boccia and Pedersen, 2001; Mezzacappa et al., 2003; Neumann et al., 1998), an open field (Stern et al., 1973), and in their defensive burying (Fernández-Guasti et al., 2001; Picazo & Fernández-Guasti, 1993).

Considerable inter-laboratory differences in testing procedures may help explain contradictions among studies comparing anxiety between virgin and postpartum rats (Lonstein, 2007). Relevant to the now four studies of postpartum rodents in the light–dark box, different strains of mice (Cook et al., 2001; Mathis et al., 1994; Rodgers and Cole, 1993) and rats (Commissaris et al., 1996; Rex et al., 1996; Schmitt and Hiemke, 1998; Shepard and Myers, 2008) differ in their anxiety. This makes it is difficult to compare results obtained from Swiss outbred mice (Maestripieri and D'Amato, 1991) with those from C57BL/6J inbred mice (Gammie et al., 2008), or between Wistar (Zuluaga et al., 2005) and Long–Evans (present study) rats. Second, the intensity of the ambient light greatly affects behavior in the light–dark box and other anxiety paradigms (Bourin and Hascöet, 2003; Hogg, 1996; Violle et al., 2009). In our pilot work, we found that differences between postpartum and diestrous virgin rats in light–dark box behavior only emerged when illumination on the light chamber was at least ~600 lx (Miller and Lonstein, 2006). Below that level, neither group found the light chamber particularly aversive. Third, the chamber in which subjects begin the light–dark box test alters their behavior (Chaouloff et al., 1997). Zuluaga et al. (2005) and Gammie et al. (2008) tested their subjects starting in the dark chamber and found no effect of postpartum state, while we and Maestripieri and D'Amato (1991) started subjects in the light chamber and found reproductive state differences. In fact, we have data showing that postpartum female rats spend less time in the light chamber if they begin the test in the dark chamber (43 ± 26 s in light) than if they are initially placed in the light chamber (165 ± 42 s in light) (Miller and Lonstein, unpublished). Fourth, whether or not females were repeatedly tested could help explain discrepancies between the studies (Bertoglio and Carobrez, 2000; Nosek et al., 2008; Rodgers and Shepard, 1993). Lastly, the stage of the estrous cycle virgins were in at testing could be relevant because some laboratories find that anxiety and fear behaviors are low when circulating ovarian hormone levels are high (Frye et al., 2000; Llaneza and Frye, 2009; Marcondes et al., 2001; Mora et al., 1996; Toufexis, 2007; Zuluaga et al., 2005). Related to this is the possibility that a few days of brief handling during vaginal smearing (typically ~10 s/day) altered the anxiety behavior of the diestous virgin females in our experiments. This remains to be examined experimentally but seems unlikely to underlie differences between our groups because both cycling females that are not smeared each day (Hard and Hansen, 1985; Toufexis et al., 1999) and smeared each day (Fleming and Luebke, 1981; Lonstein, 2005; Miller et al., 2010) are relatively anxious compared to postpartum females. Furthermore, ovariectomized female rats that are presumably not smeared each day are as anxious as cycling females smeared daily (Fernandez-Guasti and Picazo, 1992; Marcondes et al., 2001; Mora et al., 1996; Zuluaga et al., 2005).

5.2. Offspring contact influences light–dark box behavior

The hormones of pregnancy and parturition are necessary for the onset of a suite of behavioral changes in female rats (including maternal behavior, elevated aggression, anxiolysis) but these behaviors are maintained thereafter by physical interactions with offspring (Stern, 1996; Lonstein, 2005; Lonstein and Miller, 2008). This is supported by data demonstrating that postpartum ovariectomy or adrenalectomy, or prepartum hypophysectomy, have little effect on anxiety in mother rats (Hansen, 1990; Lonstein, 2005). However, recent infant contact is required for the postpartum suppression of anxiety when assessed with an elevated plus-maze (Figueira et al., 2008; Lonstein, 2005; Smith and Lonstein, 2008; Neumann, 2003). This is supported by the results from Experiment II, showing that absence of the litter for 4 h before testing increases dams' anxiety-related behaviors in a light–dark box. The importance of infant touch, rather than the hormones of pregnancy or lactation, for suppressing maternal anxiety is further evidenced by the partial blunting of fear and anxiety behaviors in nulliparous rats induced to act maternally through repeated exposure to neonates (Agrati et al., 2008; Ferreira et al., 2002). In both parous and nulliparous mothers, infant contact probably suppresses emotional behaviors through increased GABA release while females interact with the litter (Qureshi et al., 1987; Lonstein and Miller, 2008).

It is important to note that the effect of litter removal on the duration of time dams spent in the light chamber in Experiment II was only significant using a one-tailed *t*-test. We previously observed a statistically stronger effect of litter removal when dams were tested in an elevated plus maze, but comparing the present results and those of Experiment II from Lonstein (2005) that used an elevated plus-maze reveals that the magnitude of the separation effect is almost identical between studies (separated dams show ~50% decrease in the duration of time spent in the more aversive part of the apparatus). Behavior in a light–dark box and an elevated plus maze are sometimes strongly correlated (Henderson et al., 2004; Ramos, 2008), but it's also been found that the light–dark box is less sensitive than the elevated plus maze to some manipulations (Biala and Kruk; 2007; Hascöet and Bourin, 1998; McCool and Chappell, 2007; Ramos, 2008; Zuluaga et al., 2005) and this may be true for how litter contact affects their mothers' anxiety.

5.3. $GABA_A$ receptor influences on light–dark box behavior

The number of neurochemicals working together to suppress anxiety in mothers with recent contact with pups is probably vast, but it is not surprising that inhibitory GABA systems are involved. Dams and diestrous virgins do not differ in $GABA_A$ or benzodiazepine

receptor binding within the neural anxiety network (Miller and Lonstein, 2011), but central GABA release rises when dams interact with pups and falls when pups are removed (Qureshi et al., 1987). Some functional implications of this rise in GABA release are revealed by the increased freezing in response to an acoustic stimulus in dams treated with FG-7142 and decreased punished drinking in dams given PTZ (Hansen et al., 1985; Hansen, 1990).

We here expand upon these findings by demonstrating that $GABA_A$ receptor inhibition especially in postpartum female rats can, depending on the $GABA_A$ antagonist, increase anxiety-related behaviors in a light–dark box. Neither GABA site antagonism with (+)-Bicuculline, nor benzodiazepine site inverse agonism with FG-7142, greatly affected females' behavior in the light–dark box, but blocking the $GABA_A$ receptor chloride ion channel with the picrotoxin site ligand PTZ strongly impacted numerous anxiety-related behaviors and especially did so in dams. Because PTZ did not affect females' latencies to their first transition from the light to the dark chamber, its anxiogenic effect is not secondary to any gross locomotor deficits; still, the issue of whether PTZ simultaneously altered both dams' anxiety and motor activity warrants further examination because PTZ at 20–30 mg/kg doses has been seen to either have no effect on (Zienowicz et al., 2007) or reduce locomotion (File, 1984; File and Lister, 1984) in laboratory rats. In contrast to the postpartum females, we found that PTZ had little effect in diestrous virgins. This could be due to a ceiling effect in the already high-anxiety virgins, but also perhaps because dams and virgins differ in sensitivity to some anxiety-modulating drugs (see Fernández-Guasti et al., 1998, 2001; Ferreira et al., 2000).

The general ineffectiveness of (+)-Bicuculline or FG-7142 in both dams and virgins was probably not due to our choice of doses, which were in the range previously observed to affect anxiety-related behaviors in laboratory rodents (Atack et al., 2005; Evans and Lowry, 2007; File, 1984; Hansen et al., 1985; Nicolas and Prinssen, 2006; Pellow and File, 1986; Dalvi and Rodgers, 1996; Zarrindast et al., 2001). Many $GABA_A$ receptor modulators inconsistently affect anxiety in male rats (Millan, 2003) and such effects might depend on the particular behavioral tests used (Nazar et al., 1997). For example, FG-7142 can be intrinsically anxiogenic in male mice, but not when assessed with a light–dark box (Crawley et al., 1984). Similarly, we previously reported that a 4 mg/kg dose of (+)-Bicuculline is anxiogenic when dams are tested with an elevated plus-maze (Miller et al., 2010), but it was not in the present study. Lastly, while we found that PTZ is much more anxiogenic than FG-7142 in the light–dark box, it is much *less* effective than FG-7142 on dams' freezing in response to a burst of noise (Hansen et al., 1985). Such paradigm-specific effects are not unexpected and may attest to how these drugs differentially affect anxiety behavior depending on the animals' reproductive state (Fernandes et al., 1999).

PTZ binds to the picrotoxin site within the chloride channel of $GABA_A$ receptors (Bali and Akabas, 2007; Huang et al., 2001). When the picrotoxin site is bound by PTZ or similar ligands, and GABA dissociates from its binding site, the ion channel is slower to reopen (Bali and Akabas, 2007). PTZ may have had even stronger anxiogenic effects than the other antagonists we used — which competitively bind to sites outside the channel pore — because PTZ both blocks the chloride channel and slows its reopening. Because the higher dose of PTZ potently increased anxiety behavior in dams but not virgins, the picrotoxin site may deserve particular attention in future studies of how increased $GABA_A$ receptor activity contributes to the postpartum suppression of anxiety.

Acknowledgements

This research was supported in part by NICHD grant #HD057962 to JSL and a dissertation completion fellowship from Michigan State University to SMM.

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