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# Prenatal stress influences 8-OH-DPAT modulated startle responding and [$^3$H]-8-OH-DPAT binding in rats

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**Abstract**

The present study was to investigate some aspects of the 5-HT1A receptor system in adult-aged rats (50–60 days) that were either exposed to prenatal stress (PS) or not exposed to prenatal stress (CON). In the first series of experiments, rats were pretreated with vehicle, the 5-HT1A agonist 8-OH-DPAT or the 5-HT1A antagonist, WAY-100635 and exposed to 120 acoustic startle stimuli (95 dB) using a 30 s inter-trial interval. 8-OH-DPAT produced a dose-dependent increase in acoustic startle responding in CON and PS rats, with the PS rats exhibiting greater responding than CON rats. WAY-100635 depressed startle amplitudes only in the CON group. Finally, radioligand binding studies using [$^3$H]-8-OH-DPAT indicated a significant decrease in receptor density in hippocampal homogenates from PS rats but no difference in [$^3$H]-8-OH-DPAT binding from homogenates of the amygdala. Our results are consistent with earlier reports indicating that prenatal stress alters the serotonergic system. Specifically, our results indicate that gestational exposure to chronic mild stress enhances startle amplitudes following 8-OH-DPAT administration, prevents the depression in startle amplitudes following WAY-100635 administration and reduces [$^3$H]-8-OH-DPAT binding in hippocampal preparations.


*Keywords:* Gestational stress; Maternal stress; Serotonin; Hippocampus; Amygdala; Sprague–Dawley; Acoustic startle reflex; Radioligand binding

## 1. Introduction

Stressful early life experiences impact behavioral outcomes for adult-aged animals in several mammalian species, including humans and rodents (Weinstock, 1997, 2001; Kofman, 2002). In rodents, prenatal stress alters a variety of fear-related behaviors. Prenatally stressed rats spend less time in the center of an open-field (Meisel et al., 1979; Vallee et al., 1997; Lehmann et al., 2000), show increased behavioral inhibition to footshock and conditioned cues (Takahashi et al., 1992; Griffin et al., 2003), and increased defensive withdrawal (Ward et al., 2000). Alterations in neurobiological systems associated with fear-related behaviors have also been reported. For example, following footshock, plasma norepinephrine levels are greater in PS rats (Weinstock et al., 1998). Our laboratory has reported increased corticotropin-releasing hormone (CRH) content and release from amygdala minces (Cratty et al., 1995; Ward et al., 2000) as well as expansion of the lateral amygdala in adult PS rats (Salm et al., 2004). Evidence suggests that the altered neurobiology of the PS rats develops from exposure of the fetus to high plasma levels of endogenous glucocorticoids released by the mother under chronic stress (Barbazanges et al., 1996; Weinstock, 1997; Welberg and Seckl, 2001; Avishai-Eliner et al., 2002; Griffin et al., 2003). In fact, adrenalectomy of prenatally stressed dams blocks the persistent elevation of plasma corticosterone in adult PS rats after restraint stress (Barbazanges et al., 1996), implicating high levels of circulating maternal corticosterone in the long-term effects of prenatal stress in adult offspring.

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Adult and juvenile PS rats have a hyperactive hypothalamic–pituitary adrenal (HPA) axis, reflected by higher and more prolonged levels of corticosterone compared to CON rats (Peters, 1982; Henry et al., 1994; Barbazanges et al., 1996; Vallee et al., 1997; Weinstock et al., 1998; Morley-Fletcher et al., 2003). Corticosterone plays a critical role in the generation of fearful behaviors and plays an important role in the heightened fear responses of PS rats (Takahashi et al., 1990; Korte, 2001). Taken together, these behavioral and neurobiological observations form the basis of a general hypothesis that PS offspring are inherently more fearful than offspring from non-stressed pregnancies.

Serotonergic cell bodies are detectable in the brainstem of rats by gestational day 12 (Aitken and Tork, 1988), suggesting that this system could be vulnerable to the effects of prenatal stress. Indeed, previous studies found that PS rats displayed more "wet dog shakes" (i.e. serotonin syndrome) than CON rats when challenged with several doses of 5-hydroxy-L-tryptophan (Peters, 1986a). It was also reported that [$^3$H]5-HT binding was increased in cortex and decreased in hippocampus of PS rats (Peters, 1986b). The greater 5-HT receptor density in the cortex of PS rats may be due, in part, to increased 5-HT1A receptor expression, since it was previously reported that PS rats have increased levels of 5-HT1A mRNA in cortical preparations (Morley-Fletcher et al., 2004). Finally, a recent report found that the putative serotonin reuptake enhancer, tianeptine, reduced immobility time in the forced swim test in PS rats but not in CON rats (Morley-Fletcher et al., 2003). These reports support the idea that the serotonergic system is altered by prenatal stress.

5-HT1A receptors are a subclass of serotonin receptors involved in the complex phenomenon of fear-related behaviors. 5-HT1A receptors have been implicated in anxiolytic responses of the social interaction test and elevated plus maze (Dawson et al., 1995; Remy et al., 1996; Collinson and Dawson, 1997) and anxiogenic responses acoustic startle and social interaction tests (Svensson, 1985; File et al., 1996; De Almeida and Lucion, 1997) using the prototypical 5-HT1A agonist, 8-OH-DPAT (Hoyer et al., 1994). Whether the response to 8-OH-DPAT is anxiogenic or anxiolytic appears to depend on the route of administration and which receptor pool is preferentially activated. Nevertheless, it is clear that 5-HT1A receptors participate in modulating fear-related behaviors.

The present study was to investigate the effects of 8-OH-DPAT and WAY-100635 on acoustic startle responding in CON and PS rats. 8-OH-DPAT is a selective agonist for 5-HT1A receptors, having low nanomolar affinity for 5-HT1A receptors but only micromolar affinity for 5-HT7 receptors (Wood et al., 2000). The 5-HT1A antagonist, WAY-100635, has nanomolar affinity for the 5-HT1A receptor (Hoyer et al., 1994; Gozlan et al., 1995). We hypothesized that 8-OH-DPAT would increase acoustic startle responding in CON and PS rats as previously reported (Svensson, 1985; Czyrak et al., 2003). Since elevated startle responding is generally considered an anxiogenic response (Koch, 2000), we further anticipated that 8-OH-DPAT modulated startle responding in PS rats would be greater than CON rats. We hypothesized that the antagonist, WAY-100635, would depress startle responding as reported earlier (Joordens et al., 1998) and, perhaps, to a greater degree in the PS rats.

A second objective was to examine [$^3$H]-8-OH-DPAT binding in homogenates of the hippocampus and amygdala from PS and CON rats. Since PS rats have a dysregulated HPA axis resulting in higher levels of circulating glucocorticoids and systemically administered glucocorticoids decrease 5-HT1A density in the hippocampus (Takao et al., 1997; Czyrak et al., 2003), we hypothesized that 5-HT1A receptors would be reduced in the hippocampus. In the amygdala, activation of 5-HT1A receptors have been associated with anxiolytic responses (De Almeida and Lucion, 1997) and reductions in excitatory neurotransmission (Cheng et al., 1998); thus, considering the heightened fear responses of PS rats, we hypothesized a reduction in 5-HT1A receptor density in the amygdala of PS rats.

## 2. Methods

### 2.1. Animals

Male (225–250 g) and female (200–225 g) Sprague–Dawley rats were purchased from Hilltop Labs, Inc. (Hilltop, PA). All rats were maintained on a 12 h light cycle (lights on 0700 hours) with food and water available ad libitum. All rats were housed in the AAALAC approved animal quarters at the Robert C. Byrd Health Sciences Center. Finally, all procedures had the approval of the Institutional Animal Care and Use Committee (Protocol 9905–06) and were conducted in accordance with the 1996 NIH Guide for the Care and Use of Laboratory Animals.

### 2.2. Induction of prenatal stress

The prenatal stress procedure was conducted as previously described (Ward et al., 2000; White and Birkle, 2001; Griffin et al., 2003; Salm et al., 2004). Briefly, male and female rats were paired and the appearance of a vaginal plug indicated gestational day 0. The females were separated and either not manipulated (CON) or stressed (PS) once daily from gestational day 14 through birth. During the stressing procedure, the dam was removed from her home cage, placed in a new cage for enough time to give a subcutaneous saline (0.9%) injection (0.1 ml) at the nape of the neck and then returned to her cage. The occurrence of the stressing procedure was randomly timed each day during the light phase and performed by different personnel to prevent habituation. Males were weaned at

postnatal day 21 and housed as sibling pairs with free access to food and water.

### 2.3. Acoustic startle testing

Acoustic startle testing began on the male offspring (50–60 days) using 4 computer controlled startle cabinets from Med Associates, Inc. (Georgia, VT) with methods previously described (White and Birkle, 2001). The cabinets are equipped with peizo-electric transducers that convert animal movement into voltage readings. The transducers were calibrated daily according to the manufacturer's instructions. The startle stimuli consisted of 95 dB white noise bursts (50 ms duration) delivered every 30 s for 1 h. Peak startle amplitudes were recorded during a 250 ms period following noise burst onset. For the experiments shown in Figs. 1 and 3, drugs were administered such that each rat received vehicle and each of the doses in a randomized cross-over design. A washout period of 48 h occurred between sessions. After a 2 week washout period, some of the rats used to generate data in Fig. 1 were used to examine the effects of WAY-100635 on acoustic startle responding (Fig. 3). Behaviorally naïve rats were used to generate the data in Fig. 2 using a between-groups design.

### 2.4. Drug administration

8-OH-DPAT HBr (Sigma, Inc) and WAY-100635 Maleate (Sigma, Inc) were dissolved in saline (0.9%) and administered intraperitoneally (i.p.) 15 min prior to the acoustic startle test sessions. All rats were habituated to the injection procedure prior to each experiment by administering saline (0.9%, i.p.) once daily for 3 days.

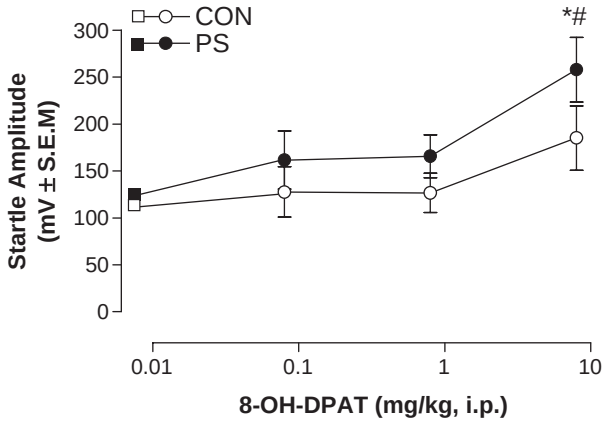


Fig. 1. Mean acoustic startle amplitudes in CON and PS rats treated with 8-OH-DPAT (0, 0.08, 0.8 and 8 mg/kg, i.p.), a 5-HT1A agonist. Vehicle treatment responses are indicated by square symbols and were not different [■ PS, □ CON]. Circular symbols represent responses to drug [● PS, ○ CON]. 8-OH-DPAT increased acoustic startle amplitudes at the highest dose in the PS rats (*$p<0.05$), but did not increase amplitudes in the CON rats. PS rats had larger amplitudes than CON rats ($^{\#}p<0.05$) at the 8 mg/kg dose ($n=11$ per group, representing 6 independent litters).

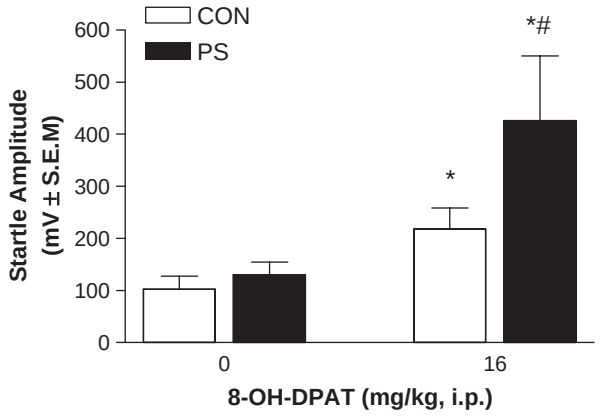


Fig. 2. Mean acoustic startle amplitudes in CON (open bars) and PS rats (closed bars) treated with vehicle or 16 mg/kg 8-OH-DPAT (i.p.). This dose significantly increased acoustic startle amplitudes in CON and PS rats (*$p<0.05$) and the increase was greater in the PS rats ($^{\#}p<0.05$) ($n=8$ per group, representing 4–5 independent litters).

### 2.5. Radioligand binding

Hippocampus and amygdala were block dissected from behaviorally naïve adult male CON and PS rats aged 50–60 days (Glowinski and Iversen, 1966) and homogenized in 3.5 ml of 320 mM sucrose and 5 mM Tris–HCl at pH 7.4. The homogenates were centrifuged at 100 $\times g$ for 10 min at 4 °C. The supernatant was centrifuged at 70,000 $\times g$ for 25 min at 4 °C and the pellet resuspended in 10 volumes of 50 mM Tris–HCl at pH 7.4. Aliquots were frozen at −80 °C. For radioligand binding, aliquots of membranes (50–80 μg) were incubated for 30 min at room temperature with 50 mM Tris–HCl (pH 7.4) containing various concentrations of $^{3}$H-8-OH-DPAT (0.10–50 nM) (NEN Dupont, Inc). The reaction was terminated by rapid filtration over Whatman GF/B filters followed by 3 washes with 3.5 ml of ice-cold buffer. Non-specific binding was determined in the presence

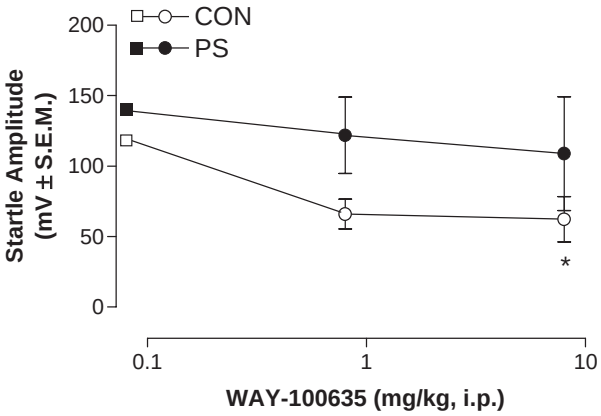


Fig. 3. Mean acoustic startle amplitudes in CON and PS rats treated with WAY-100635 (0, 0.8 and 8 mg/kg, i.p.), a 5-HT1A antagonist. Vehicle treatment responses are indicated by square symbols and were not different [■ PS, □ CON]. Circular symbols represent responses to drug [● PS, ○ CON]. WAY-100636 depressed startle amplitudes at the highest dose in the CON rats (*$p<0.05$) but did not have an effect on startle amplitudes in the PS rats ($n=6$ per group, representing 3 independent litters).

Table 1

| Region | Group[#] | [$^3$H]-8-OH-DPAT binding | |
|---|---|---|---|
| | | $B_{max}$ (fmol/mg prot) | $K_D$ (nM) |
| Hippocampus | PS | 105.2±11* | 4.5±1.4 |
| | CON | 139.0±13 | 5.8±1.5 |
| Amygdala | PS | 50.2±5 | 4.5±1 |
| | CON | 48.1±15 | 6.9±4 |

Values are means±S.E.M. [#]$n$=5–6 per group, representing 2–3 independent litters. *Different from CON ($p$<0.05).

of 10 μM 5-HT HCl. Protein concentrations of the final dilution used in the assay were determined (Lowry et al., 1951).

### 2.6. Data analysis

The primary data for the acoustic startle experiments were peak startle amplitudes following onset of the noise burst. The acoustic startle data were not normally distributed (Shapiro–Wilk test, $p$<0.05) and further analysis was conducted using Newman–Keuls Analog for Multiple Comparisons Test for the experiments shown in Figs. 1–3. The primary data for the radioligand binding experiments were $B_{max}$ (fmol/mg protein) and $K_D$ (nM), shown in Table 1. The radioligand binding data were analyzed using non-linear regression analysis in order to calculate $B_{max}$ and $K_D$ for individual rats. Group means were compared by Student's $t$-test. The acoustic startle data was analyzed using GB-STAT® Version 7 and the radioligand binding data was analyzed using GraphPad Prism® Version 3.

## 3. Results

### 3.1. Acoustic startle responding

Fig. 1 shows the effects of 0–8 mg/kg 8-OH-DPAT on acoustic startle amplitudes. Examination of the acoustic startle amplitude within session at the 8 mg/kg dose indicated that the greatest elevation occurred during the first 30 min of the session, relative to the responses measured after vehicle administration, and subsequent analysis was restricted to this time period. Using the Newman–Keuls analog test, comparison of startle amplitudes after vehicle administration indicated that startle amplitudes were not different between PS and CON rats ($t$<0.5). Further analysis found that, although there was a trend for elevated startle amplitudes in the CON group, none of the doses of 8-OH-DPAT significantly elevated startle amplitudes above those measured after vehicle administration (all $t$<2.2). In the PS group, only the highest dose (8 mg/kg) significantly elevated startle amplitudes above those measured after vehicle administration ($t$=4.3963, $p$<0.05). Moreover, at the 8 mg/kg dose, the elevation in startle amplitudes was significantly greater in the PS rats than the CON rats treated with the same dose ($t$=2.5788, $p$<0.05).

The lack of statistically significant elevations of startle amplitudes in the CON rats prompted the use of a larger dose of 8-OH-DPAT for further comparison. Using a between-groups design in behaviorally naïve rats, we examined the effects of 16 mg/kg 8-OH-DPAT. Again, startle amplitudes after vehicle administration were similar ($t$<1.9). Newman–Keuls analog test found that 8-OH-DPAT increased startle amplitudes in both groups (both $t$>3.5, $p$<0.05). Furthermore, it was found that startle amplitudes were greater in the PS rats than the CON rats after administration of 16 mg/kg ($t$=2.9397, $p$<0.05). Neither higher doses nor more rats were tested due to the high likelihood of 8-OH-DPAT-induced toxicity (Hjorth, 1985; Evenden and Angeby-Moller, 1990; Bill et al., 1991; Blanchard et al., 1993).

Fig. 3 shows the results of WAY-100635 on acoustic startle amplitudes in PS and CON rats. Examination of the within session time course of these data indicated that a depression in acoustic startle responses occurred during the last 30 min of the session, relative to the vehicle control, and subsequent analysis was performed on data from this time period. As noted above, startle amplitudes following vehicle administration were not different between PS and CON rats ($t$<0.2). The highest dose of WAY-100635 depressed startle amplitudes in the CON rats relative to the vehicle control ($t$=2.8674, $p$<0.05). However, at either dose, startle amplitudes were not depressed in the PS rats (both $t$<1.6).

### 3.2. Radioligand binding

Table 1 shows the results of the radioligand binding studies. In the hippocampus, the PS rats had a significant decrease in $B_{max}$ when compared to the CON rats ($t$= 2.075, $p$=0.0422), indicating reduced receptor density in the hippocampus of PS rats. Analysis of the $K_D$ values in hippocampal homogenates indicated they were not significantly different ($t$=0.6388, $p$>0.5). In the amygdala samples, differences between the groups were not detected in either measure ($B_{max}$: $t$=0.1297, $p$> 0.8; $K_D$: $t$= 0.5692, $p$>0.5). The values calculated for $B_{max}$ and $K_D$ are in agreement with previously published data for the hippocampus and amygdala (Chalmers and Watson, 1991; Nenonene et al., 1994; Popova et al., 1998).

## 4. Discussion

We found that administration of a 5-HT1A agonist, 8-OH-DPAT, dose dependently elevated acoustic startle amplitudes in PS and CON rats. PS rats showed larger elevations in startle amplitudes at the two highest doses compared to the CON rats. On the other hand, the 5-HT1A antagonist, WAY-100635, depressed startle amplitudes in the CON rats but not the PS rats. In addition, using radioligand binding techniques, we found that specific binding of [$^3$H]-8-OH-DPAT to hippocampal homogenates

was significantly reduced in the PS rats compared to CON rats, but that [$^3$H]-8-OH-DPAT binding was unchanged in the amygdala of PS rats.

Consistent with our previous report (White and Birkle, 2001), baseline (i.e. vehicle treatment) acoustic startle responding was not different between CON and PS rats. Upon administration of 8-OH-DPAT, there was a dose-dependent increase in acoustic startle amplitudes as previously found (Svensson, 1985; Czyrak et al., 2003). The increase in startle amplitudes was larger in the PS rats at the two highest doses. Although the maximum dose–response function was not defined in these experiments, the data suggest a leftward shift in the dose–response function for the PS rats. The dose-dependent elevation in acoustic startle amplitudes by 8-OH-DPAT would be considered an anxiogenic response (Koch, 2000) and larger increases in startle amplitudes are consistent with the hypothesis that PS rats are more fearful.

It is uncertain what mechanism may account for the enhanced startle amplitudes of the PS rats after administration of 8-OH-DPAT. An intriguing possibility is suggested by a report showing lesions of the raphe nuclei lead to elevated acoustic startle responding (Davis and Sheard, 1974) and the implication is that intact, serotonergic cell bodies projecting from the raphe nuclei exert an inhibitory influence on startle responding. Since the 5-HT1A receptors in the dorsal raphe function as autoreceptors to reduce serotonergic neuronal activity (Fletcher et al., 1993), it is reasonable to hypothesize that administration of 8-OH-DPAT reduces neuronal activity in the raphe nuclei, leading to elevated startle amplitudes. That this effect is more pronounced in the PS rats would indicate that the 5-HT1A receptor system is more sensitive to modulation by 8-OH-DPAT in this group. Clearly, more experiments are needed to strengthen this hypothesis. Finally, given that 8-OH-DPAT was administered systemically, the possible involvement of 5-HT7 receptors, for which 8-OH-DPAT has partial agonist activity (Wood et al., 2000), cannot be ruled out. The influence of 5-HT7 receptors on acoustic startle responding is unknown.

The 5-HT1A antagonist, WAY-100635, depressed startle responding in the CON rats but not in the PS rats. The depression of startle amplitudes in the CON rats is consistent with an earlier report in which WAY-100635 depressed both baseline and potentiated startle amplitudes in Wistar rats (Joordens et al., 1998). However, the reason for this depression is unknown since evidence indicates that administration of WAY-100635 does not produce measurable responses in several behavioral and physiological paradigms related to 5-HT1A receptor function (Forster et al., 1995; Gozlan et al., 1995; Fletcher et al., 1996). Instead, the present results may support the idea that WAY-100635 interacts with neurotransmitter systems, such as the dopaminegic system, to reduce startle amplitudes (Jackson et al., 1998; Joordens et al., 1998). Previous studies indicated that activation of D1- and D2-like receptors increase startle responding (Naudin et al., 1990; Svensson, 1990). Moreover, it has been demonstrated that prenatal stress alters dopamine turnover, dopaminergic laterality and dopaminergic interhemispheric coupling (Fride and Weinstock, 1987, 1988, 1989). Although speculative, these studies suggest that differences between CON and PS rats on acoustic startle responding after treatment with WAY-100635 could be due, at least in part, to differences in dopaminergic modulation of the startle response.

In the radioligand binding experiments we found that [$^3$H]-8-OH-DPAT binding was reduced in the hippocampus of PS rats, indicating that prenatal stress was associated with reduced 5-HT1A receptor density in this region. Our finding is consistent with reduced [$^3$H]5-HT binding in the hippocampus of PS rats found previously (Peters, 1986b) and provides specific information about what portion of the serotonin receptor pool is reduced in PS rats. Additionally, the 30% reduction in receptor density we report is consistent with the 32% reduction in dendritic spine density in the hippocampus of PS rats reported earlier (Hayashi et al., 1998) and suggests that reduced 5-HT1A receptor density is secondary to reduced dendritic spine density.

The reason for reduced 5-HT1A receptor density in the hippocampus of PS rats was not addressed by this study. However, the hippocampus is particularly susceptible to stress-induced damage, evidenced by findings such as reduced synaptic density (McEwen et al., 1992; Magarinos et al., 1997; McEwen and Magarinos, 1997). In addition, it was previously reported that systemic administration of corticosterone reduces 5-HT1A receptor density in the hippocampus of rats (Takao et al., 1997; Czyrak et al., 2003). Furthermore, PS rats are known to have greater stress-induced elevations of corticosterone than CON rats (Peters, 1982; Henry et al., 1994; Barbazanges et al., 1996; Vallee et al., 1997; Weinstock et al., 1998). Taken together, these reports suggest that the greater levels of circulating corticosterone may be the ultimate cause of reduced 5-HT1A receptor density in the hippocampus of PS rats relative to the CON rats.

We did not find a difference in [$^3$H]-8-OH-DPAT binding in homogenates of the amygdala, indicating that 5-HT1A receptor density was not affected by prenatal stress in this region. In addition, the results imply that the same mechanism serving to reduce 5-HT1A receptor density in the hippocampus of PS rats is not operative in the amygdala. Previous reports from our laboratory identified neurobiological changes in the amygdala associated with prenatal stress. These changes include increased corticotropin-releasing hormone content and release (Cratty et al., 1995) as well as an increased numbers of neurons and glia specifically in the lateral amygdala of PS rats (Salm et al., 2004). Although we hypothesized a decrease in 5-HT1A receptor density based on the fearful behavioral phenotype of PS rats, given our previous finding of increased cellular population in the lateral amygdala, we could have found an increase in 5-HT1A receptor density. However, block

dissection of tissue in the current study did not allow examination of [$^3$H]-8-OH-DPAT binding in specific subregions of the amygdala or immediately adjacent regions. In fact, a previous study found that chronic administration of corticosterone reduced 8-OH-DPAT specific binding in the entorhinal cortex (Czyrak et al., 2003), a region included in block dissections containing the amygdala. Therefore, the possibility remains open that there is differential expression of 5-HT1A receptors within the amygdala and surrounding regions in the brains of PS rats.

In conclusion, our findings extend earlier studies of the serotonergic system by examining the 5-HT1A receptor subtype. Using a relatively selective agonist for 5-HT1A receptors, 8-OH-DPAT, we found an enhancement of acoustic startle responding in PS rats relative to CON rats. Interestingly, the antagonist WAY-100635 reduced startle amplitudes in the CON rats but not the PS rats. Finally, we found that 5-HT1A receptor density was reduced in the hippocampus of PS rats but not in the amygdala.

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