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Prenatal stress, regardless of concurrent escitalopram treatment, alters behavior and amygdala gene expression of adolescent female rats

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ABSTRACT

Depression during pregnancy has been linked to *in utero* stress and is associated with long-lasting symptoms in offspring, including anxiety, helplessness, attentional deficits, and social withdrawal. Depression is diagnosed in 10–20% of expectant mothers, but the impact of antidepressant treatment on offspring development is not well documented, particularly for females. Here, we used a prenatal stress model of maternal depression to test the hypothesis that *in utero* antidepressant treatment could mitigate the effects of prenatal stress. We also investigated the effects of prenatal stress and antidepressant treatment on gene expression related to GABAergic and serotonergic neurotransmission in the amygdala, which may underlie behavioral effects of prenatal stress. Nulliparous female rats were implanted with osmotic minipumps delivering clinically-relevant concentrations of escitalopram and mated. Pregnant dams were exposed to 12 days of mixed-modality stressors, and offspring were behaviorally assessed in adolescence (postnatal day 28) and adulthood (beyond day 90) to determine the extent of behavioral change. We found that *in utero* stress exposure, regardless of escitalopram treatment, increased anxiety-like behavior in adolescent females and profoundly influenced amygdala expression of the chloride transporters KCC2 and NKCC1, which regulate GABAergic function. In contrast, prenatal escitalopram exposure alone elevated amygdala expression of 5-HT_{1A} receptors. In adulthood, anxiety-like behavior returned to baseline and gene expression effects in the amygdala abated, whereas deficits emerged in novel object recognition for rats exposed to stress during gestation. These findings suggest prenatal stress causes age-dependent deficits in anxiety-like behavior and amygdala function in female offspring, regardless of antidepressant exposure.

1. Introduction

The impact of maternal depression and its treatment on offspring neurodevelopment is of great consequence to human health. Depression affects approximately 11% of expectant mothers (Gaynes et al., 2005) and a disproportionate percentage of women in the general population (Mitchell et al., 2011; Parker and Brotchie, 2010). Despite these findings, few preclinical studies have documented the impact of clinically relevant *in utero* antidepressant exposure on offspring, and fewer have studied the impact on female offspring (Bourke et al., 2014).

In utero exposure to antidepressants may mitigate the effects of depression-related gestational stress or may directly influence offspring development. Prenatal stress is associated with later-life symptoms of anxiety, helplessness, attentional deficits, and social withdrawal in both humans and animal models (Bourke et al., 2013a; Frye and Wawrzycki, 2003; Mueller and Bale, 2007;

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Newport et al., 2002). Emotional deficits caused by prenatal stress are often sex-dependent (Mueller and Bale, 2007; Tibu et al., 2014; Van den Hove et al., 2013), and sex may also modulate the effects of *in utero* antidepressant exposure.

The impact of prenatal stress on offspring behavior is mediated in part by changes to the basolateral nucleus of the amygdala (BLA), a brain structure involved in emotional perception and behavior (Ledoux, 2004; Pape and Pare, 2010; Walker and Davis, 2008). A wealth of evidence has correlated prenatal stress effects in the BLA with elevated anxiety-like behavior (Buss et al., 2012; Cratty et al., 1995; Kraszpulski et al., 2006; Sadler et al., 2011). Furthermore, prenatal stress alters amygdala neuron excitability (Ehrlich and Rainnie, 2015) and gene expression related to GABA (Laloux et al., 2012; Sarro et al., 2014), an inhibitory neurotransmitter that tightly regulates amygdala function and affective state (Ehrlich et al., 2009; Quirk and Gehlert, 2003; Rainnie et al., 2004).

Signaling by the fast GABA receptor, $GABA_A$, is modulated the by the intracellular concentration of the receptor's main permeant ion, chloride. The concentration gradient of chloride across neuronal membranes, which dictates the strength and sign of GABAergic synaptic currents, is established by chloride transporters that accumulate or extrude chloride ions. During postnatal development, BLA neurophysiology is highly dynamic (Ehrlich et al., 2012; Thompson et al., 2008), as neurons reduce expression of the chloride accumulator, NKCC1 (Na—K—Cl cotransporter 1), in favor of the chloride extruder, KCC2 (K—Cl cotransporter 2), switching $GABA_A$ receptors from excitatory to inhibitory (Ehrlich et al., 2013). In adults, stress influences GABAergic transmission by regulating the balance NKCC1 and KCC2 expression (Maguire, 2014; Sarkar et al., 2011). Despite the stress sensitivity of these transporters and their role in neuronal maturation, it is unknown whether prenatal stress or escitalopram influences their expression in the developing amygdala.

Here, we examined effects of prenatal antidepressant exposure and its interaction with prenatal stress on developing female offspring. We used a rodent prenatal stress model of maternal depression (Bourke et al., 2013a, 2013b) in which dams are continuously exposed to clinically-relevant doses of escitalopram, a selective serotonin reuptake inhibitor, throughout gestation concurrent with chronic, unpredictable mild stress. The effects of this paradigm on maternal behavior (Bourke et al., 2013a) and male offspring (Bourke et al., 2013b) have previously been reported. Here we investigated the age-dependent effects of prenatal stress and/or chronic *in utero* antidepressant exposure on behavior and chloride transporter expression in the BLA during adolescence and adulthood on female offspring. We hypothesized that anxiogenic effects of prenatal stress would reflect dampened GABAergic function in the BLA, and such effects would be mitigated by prenatal antidepressant exposure. Given that escitalopram may help treat depression via regulation of serotonergic transmission in the amygdala (Arnone et al., 2012; Bigos et al., 2008; Lanzenberger et al., 2012; Rosenblau et al., 2012), we also measured offspring expression of two amygdala serotonin receptors linked to the treatment of depression, *5-Ht$_{1a}$* and *5-Ht$_7$* (Bosker et al., 2001; Hahn et al., 2010; Naumenko et al., 2014; Takeda et al., 2005). The collected data indicate that *in utero* exposure to chronic stress, regardless of escitalopram treatment, caused temporary behavioral and gene expression deficits during adolescence that resolved by adulthood.

2. Methods

2.1. Prenatal stress and escitalopram exposure

Rats used in this experiment were bred in-house from male Sprague—Dawley retired breeders and nulliparous females weighing 200—225 g, purchased from Charles River Laboratories (Charles River, Wilmington, MA). Both the stress paradigm and drug administration paradigms have been previously characterized (Bourke et al., 2013a, 2013b). Nulliparous female rats were implanted with Alzet 28-day osmotic minipumps (model 2ML4, Alzet, Cupertino, CA) delivering either 0.9% saline or escitalopram oxalate in 0.9% saline based upon the expected weight of the pregnant dam on gestational day (G) 21 (Bourke et al., 2013a, 2013b). The estimated expected weight was based on assessment of G21 weights from 4 previous studies ($n = 36$). For these studies, the actual dose on G21 was 12.2 mg/kg/day. This results in steady-state serum drug concentrations that are always within the clinically observed range even though the dose is slightly higher early in the experiment prior to the weight gain associated with pregnancy (Bourke et al., 2013a). Escitalopram oxalate was generously provided by Lundbeck USA (Paramus NJ). Three days after minipump implantation, females were bred with retired breeder males. Gestational day 0 was established as the day when a sperm plug was noted. On G9, the chronic unpredictable mild stress model of depression began and consisted of restraint, cage tilt, damp bedding, cage changes, noise, and overnight illumination (Bourke et al., 2013a). Prenatal stress began on G9 because it corresponds with development of the fetal central nervous system (Clancy et al., 2001) and minimizes premature termination of the pregnancy as a result of excessive stress. The final prenatal stress exposure occurred on G20.

2.2. Animals

All offspring were kept on a 12:12 light:dark cycle (lights on at 7:00 AM) in a humidity (60%) and temperature (20°C—23 °C) controlled facility with their natural mother. We (Bourke et al., 2013a) have reported that neither this stressor paradigm nor escitalopram treatment modifies gestational length, litter size, or maternal care of the offspring. Rodent diet 5001 chow (Purina Mills, Richmond, IN) and water were available *ad libitum* throughout the study. Three days after birth, rat pups were sexed, and litters were culled to six male and two female pups per litter. Only female rats were used for this study; male offspring were allocated to a parallel study following weaning (Bourke et al., 2013b). Animals were weaned on post-natal day (PND) 21 and kept in same-sex pairs. Only one pup from a litter was assigned to an endpoint in order to prevent litter effects (Holson and Pearce, 1992). Each group was assigned between 8 and 12 pups. Experimental groups included non-stress/saline (Control), non-stress/escitalopram (Escit), stress/saline (Stress), and stress/escitalopram (Stress + Escit) with assessment occurring either during adolescence or in adulthood. All experiments were performed in accordance with the Institutional Animal Care and Use Committee of Emory University and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

2.3. Behavior

Although rodents cannot be queried regarding their affective states, behavioral tests have been established which probe circuitry implicated in the pathogenesis of depression and anxiety (Crawley, 2007; Whishaw and Kolb, 2005). Behavioral testing was conducted between PND 28 and 35 for the adolescent endpoints with the administration of one test per day with the exception of the sucrose consumption test which lasted 48 h in the animals' home cage. An additional cohort of rats with identical rearing was used to assess behavior in adulthood (Control $n = 12$; Escit $n = 11$; Stress $n = 15$; Stress + Escit $n = 10$). For the adult endpoint, rats were maintained in the colony as described until at least PND 90, and then, adult behavior was assessed. Behaviors were recorded and scored using Cleversys Top Scan/Forced Swim software (Reston, VA). All behavioral tests and scoring were conducted by an experimenter blind to treatment groups. Behavioral equipment was cleaned with 70% ethanol between animals.

2.3.1. Open field

Rats were placed in the center of a 70 × 70 cm^2 arena and were allowed to explore for 10 min. Overall distance traveled as well as the time spent in the center of the arena versus the periphery was used as a metric of anxiety-like behaviors (Prut and Belzung, 2003). This test was conducted in both the light and dark cycles in order to assess behavior both during the dormant and active phases, respectively, of the rat. In the light cycle condition, testing was conducted under bright overhead illumination by fluorescent lighting. In the dark cycle condition, testing was conducted under dim red light.

2.3.2. Social interaction

Rats were again placed in the center of a 70 × 70 cm^2 arena for assessment of social interaction. In this particular test, the arena contained an age-matched novel female stimulus conspecific. Experimental animals were allowed to explore the arena for 10 min and latency to interact with the novel animal as well as total time interacting with the novel animal were measured. Testing took place during the light cycle under bright light.

2.3.3. Novel object recognition

Rats were placed in the center of a 70 × 70 cm^2 arena containing two identical objects. Following a 1 h delay, rats were again placed in the arena with one familiar object (from previous exposure) and a novel object. Time spent investigating the novel object was measured as an index of object recognition memory (Ennaceur and Delacour, 1988). The initial object exposure lasted 15 min, and the recorded object recognition session lasted 5 min and took place during the light cycle.

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2.3.4. Elevated plus maze

The elevated plus maze consists of a "+" shaped maze with two open arms and two closed arms elevated 1 m off the ground. Total arm entries as well as time spent in the open versus closed arms were recorded with the latter used as a metric of anxiety-like behavior (Walf and Frye, 2007). In addition, risk assessment behaviors were tabulated as an additional metric of anxiety-like behavior (Whishaw and Kolb, 2005). The test lasted for 5 min and was conducted during the animals' dark cycle.

2.4. Gene expression

Animals were killed by decapitation under isoflurane anesthesia on PND 42 (adolescents) or PND 90–120 (adults), and the brains were quickly removed. To quantify the expression of chloride transporter and serotonin receptor transcripts in isolated basolateral amygdala (BLA) tissue from adolescents, 350 μm coronal slices containing the BLA were prepared as previously described (Ryan et al., 2012). The BLA from each slice was excised immediately by microdissection. For adult rats, following decapitation brains were immediately frozen on dry ice and stored at −80 °C until dissection. The BLA was dissected using a 1 mm micropunch tool and subsequently stored until RNA extraction. For adolescent samples, BLA samples were homogenized in Trizol (Invitrogen, Carlsbad, CA), and isolated RNA was reverse transcribed with a cocktail containing 5 μl of 10x RT buffer, 10 mM dNTP mix, 10x random hexanucleotides, Multiscribe RT 5 U/μl, and RNAase free water. For adult samples, BLA samples were homogenized in Trizol (Invitrogen, Carlsbad, CA), and isolated RNA was reverse transcribed with a cocktail containing 2 μl of 10x RT buffer, 8 mM dNTP mix, 10x random hexanucleotides, Multiscribe RT 5 U/μl, and RNAase free water. cDNA was stored at −20 °C. All reagents were obtained from Applied Biosystems (Foster City, CA). All reactions were prepared in triplicate using a 40 cycle thermal cycling program. Measured primers include *Nkcc1* (Taqman ID: Rn00582505_m1), *Kcc2* (Rn00592624_m1), and *5Ht1a* (Rn00561409_s1). Real-time PCR reactions were performed using an Applied Biosystems 7500 Fast-Real Time PCR system (Applied Biosystems, Foster City, CA). All mRNA measurements were normalized to *18S* (Hs99999901_s1) rRNA expression. We calculated fold change using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and presented fold changes for each experimental group normalized to average values for the control group.

2.5. Hormone assessments

A separate cohort of rats with identical rearing was used to measure serum corticosterone and estradiol concentrations. For corticosterone measures, tail snip blood was collected after a restraint stress challenge (30-min session in an acrylic rat restrainer; BrainTree Scientific, Braintree, MA) on PND 38. Plasma corticosterone was assessed using the ImmunoChem ^{125}I Corticosterone RIA kit with a sensitivity of 1 ng/mg (MP Biomedicals, Orangeburg, NY). On PND 42, rats were rapidly decapitated, and trunk blood was collected for estradiol assessment (DSL-4400 RIA Kit with a sensitivity of 4.7 pg/mL; DSL, Webster, TX). All samples were run in duplicate.

2.6. Data analysis

Behavior and hormone assay data were compared using two-way ANOVA with the factors of stress (control vs stress) and drug (vehicle vs escitalopram) following assessment of equal variance and normality. Gene expression data were analyzed as described above, and compared using two-way ANOVA. Data were considered significant when $p < 0.05$. All data are expressed as mean ± the standard error of the mean (S.E.M.).

3. Results

3.1. Prenatal escitalopram treatment did not rescue the anxiogenic effect of prenatal stress during adolescence

We first determined whether prenatal escitalopram could modulate the behavioral effects of prenatal stress (Fig. 1). When testing occurred during the animals' dark cycle, prenatal stress reduced the time adolescent female offspring spent in the center of the open field (main effect of prenatal stress, $F_{1,28} = 14.91$, $p < 0.05$; Fig. 1A). In contrast, prenatal escitalopram had no effect on open field behavior ($p > 0.05$ for main effect of drug and interaction between stress and drug). Overall mobility (distance traveled) did not differ among groups ($p > 0.05$; Fig. 1B). When testing occurred during the animals' light cycle, no differences in open field behavior were detected ($p > 0.05$; Supplemental Fig. S1). Importantly, these effects of prenatal stress were limited to adolescence, because no differences in time spent in field center or total distance traveled were observed in adulthood ($p > 0.05$; Fig. 1C and D).

Elevated plus maze activity revealed similar effects. Although no differences were detected in the total number of elevated plus arm entries over the 5 min period ($p > 0.05$; Supplemental Fig. S2), offspring exposed to prenatal stress exhibited more risk-assessment behavior. Rats exposed to stress *in utero* showed a higher degree of risk-assessment behavior as evidenced by a greater number of stretch attend postures (main effect of stress: $F_{1,27} = 9.95$, $p < 0.05$) relative to non-stressed animals (10.50 ± 1.8 and 5.62 ± 1.39 postures, respectively). In addition, *in utero* escitalopram exposure also increased stretch attend postures (Escit: 8.57 ± 1.01; Stress + Escit: 15.13 ± 2.47 postures), producing a main effect of drug treatment ($F_{1,27} = 4.37$, $p < 0.05$) when compared to vehicle-exposed rats.

3.2. Prenatal escitalopram reduced social interaction in adolescent females

We also tested the effects of prenatal stress and escitalopram on social interaction in adolescent females (Fig. 2A). Exposure to escitalopram *in utero* reduced social interaction in adolescence as evidenced by a main effect of drug on social behavior ($F_{1,28} = 4.61$, $p < 0.05$; 43.50 ± 3.90 interactions for Escit vs. 53.50 ± 2.24 interactions for Controls). The impact of prenatal stress exposure on social interaction was less robust; although the effect was directionally similar, the difference did not reach statistical significance ($F_{1,28} = 3.91$, $p = 0.06$). However, it is important to note that the power of this particular comparison was below the desired level of 0.8; therefore, although the null cannot be rejected in this particular case, it is possible that a significant effect of prenatal stress would be documented with a larger sample size. There was no interaction between the two conditions ($p > 0.05$). Consistent with the resolution of the anxiogenic effects of prenatal stress by adulthood, no effects of prenatal stress or escitalopram on social interaction were detected in adulthood (Fig. 2B; $p > 0.05$ in all cases).

3.3. Prenatal escitalopram did not rescue prenatal stress-induced deficits in novel object recognition in adult females

We subsequently tested the effects of prenatal stress and escitalopram on memory for familiar objects following a 1 h delay after initial exposure (Fig. 3A). Consistent with previous literature, control females did not exhibit a preference for novel objects during adolescence. The adolescent control females investigated the novel object 51.59 ± 6.36% of the time, and therefore, were operating at chance (Fig. 3A). Prenatal stress and escitalopram treatment did not affect performance in adolescence as revealed by a two-way ANOVA ($p > 0.05$ in all cases).

The deficit in novel object recognition was overcome with age as control females investigated the novel object well above chance (70.58 ± 6.84%) in adulthood (Fig. 3B). Importantly, prenatal stress prevented the emergence of novel object recognition because adult female offspring previously exposed to prenatal stress still investigated around chance levels (46.85 ± 7.87%; main effect of stress: $F_{1,42} = 4.12$, $p < 0.05$). In contrast, prenatal escitalopram exposure did not impair novel object recognition in adulthood ($p > 0.05$) and prenatal stress and escitalopram did not interact to differentially alter investigation beyond the main effect of stress alone ($p > 0.05$).

3.4. Prenatal stress decreased expression of Kcc2 and increased expression of Nkcc1 in amygdala of adolescent offspring

Next, we investigated changes to amygdala gene expression that could account for the observed behavioral effects of prenatal stress and escitalopram. BLA excitability is regulated by GABAergic tone and chloride transporter function, and we found that prenatal stress decreased *Kcc2* expression and increased *Nkcc1* expression during adolescence. Prenatal stress exposure decreased *Kcc2* mRNA

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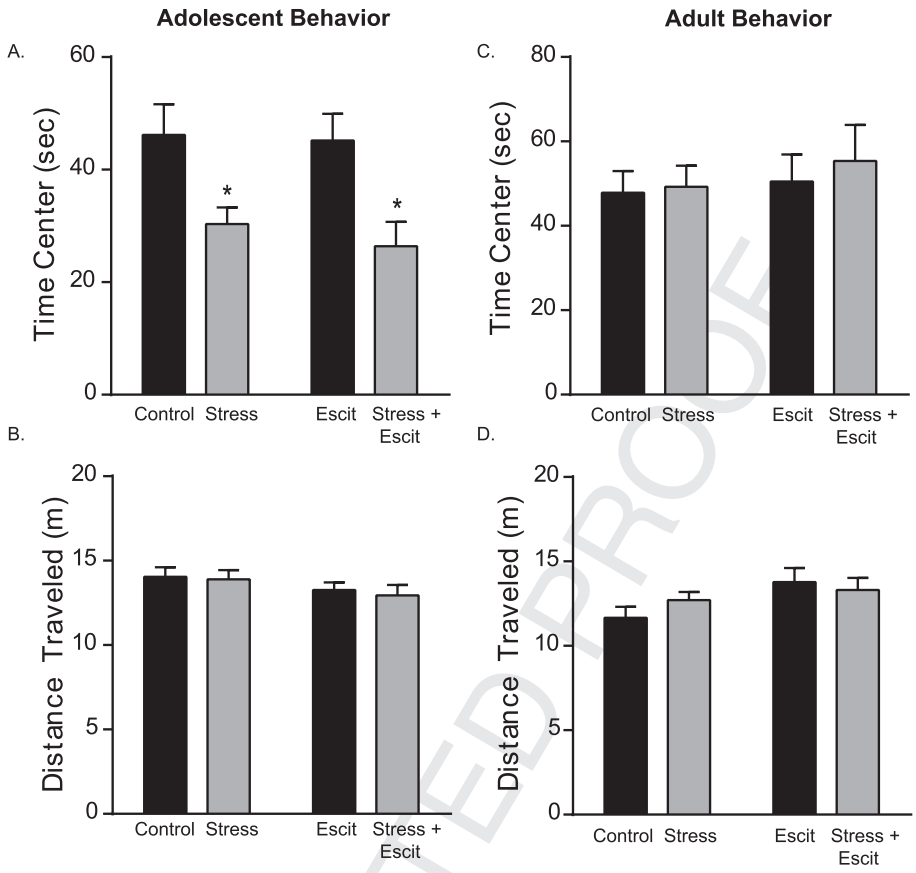

Fig. 1. Anxiety-like behavior of adolescent, female offspring exposed *in utero* to stress and/or escitalopram. *A, B*) Rats were allowed to explore an open field for 10 min. Prenatal stress reduces central tendency regardless of escitalopram treatment (A). No differences in mobility were detected as evidenced by equivalent total distance traveled (B). No differences were observed in time spent in the center (C) or mobility (D) in adults. For all, * indicates $p < 0.05$ and error bars indicate standard error of the mean (S.E.M.).

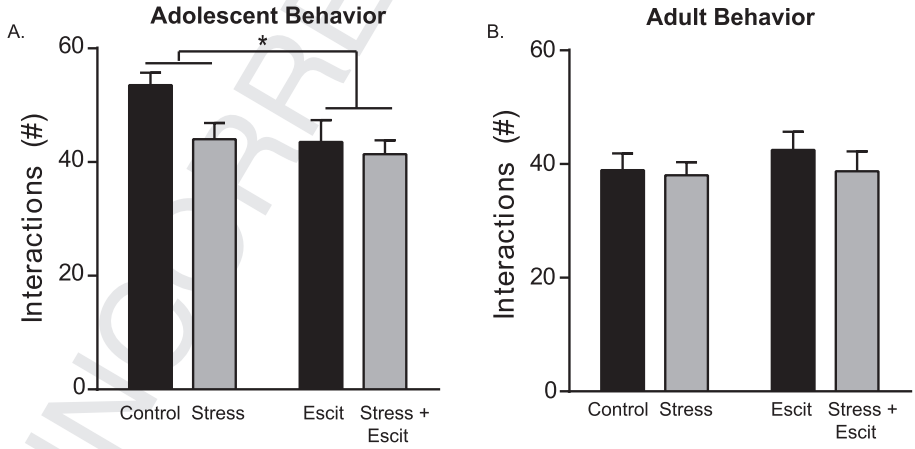

Fig. 2. Effects of prenatal stress and/or escitalopram on social interaction in adolescent and adult female offspring. *A*) Prenatal escitalopram reduced social interaction with a novel conspecific of the same age and sex regardless of prenatal stress exposure. Prenatal stress did not have an independent main effect on this behavior ($p = 0.06$). *B*) These behavioral effects were transient and were not documented in adult offspring. For all, * indicates $p < 0.05$ and error bars indicate standard error of the mean (S.E.M.).

levels by 70% in the BLA (Fig. 4A; main effect of stress: $F_{1,28} = 8.80$, $p < 0.05$). Prenatal escitalopram did not independently alter expression of *Kcc2* ($p > 0.05$ for main effect of drug); however, prenatal stress interacted with escitalopram ($F_{1,28} = 6.71$, $p < 0.05$). Expression of *Nkcc1* mRNA increased by 2.2-fold in response to prenatal stress (Fig. 4B, main effect of stress: $F_{1,28} = 20.40$, $p < 0.05$;), but was not independently impacted by prenatal escitalopram (main effect of drug: $p > 0.05$). The combination of prenatal stress and escitalopram again had a similar effect to prenatal stress alone in adolescence, increasing *Nkcc1* mRNA 2-fold (interaction: $F_{1,28} = 5.69$, $p < 0.05$).

By adulthood, the effects of prenatal stress on chloride transporter expression had abated. No effects of prenatal stress, escitalopram, or the combination of the two were observed on *Kcc2* ($p > 0.05$ in all cases) or *Nkcc1* expression ($p > 0.05$ in all cases) in the BLA of adult offspring (Table 1).

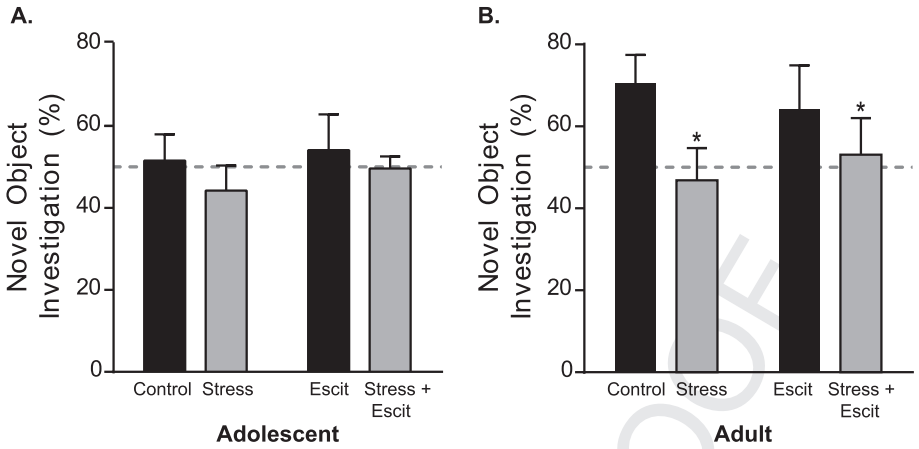

Fig. 3. Effects of prenatal stress and/or escitalopram on novel object recognition in adolescent and adult female offspring. A) One hour following pre-exposure, adolescent females showed no preference for the novel object, regardless of *in utero* condition. Rats investigated the novel object around chance levels (dashed line) in each group. *B*) When identically tested in adulthood, control female offspring now exhibit a preference for the novel object. Prenatal stress exposure, in the absence or presence of concurrent prenatal escitalopram exposure, prevented novel object recognition, causing investigation at chance levels. For all, * indicates $p < 0.05$ and error bars indicate standard error of the mean (S.E.M.).

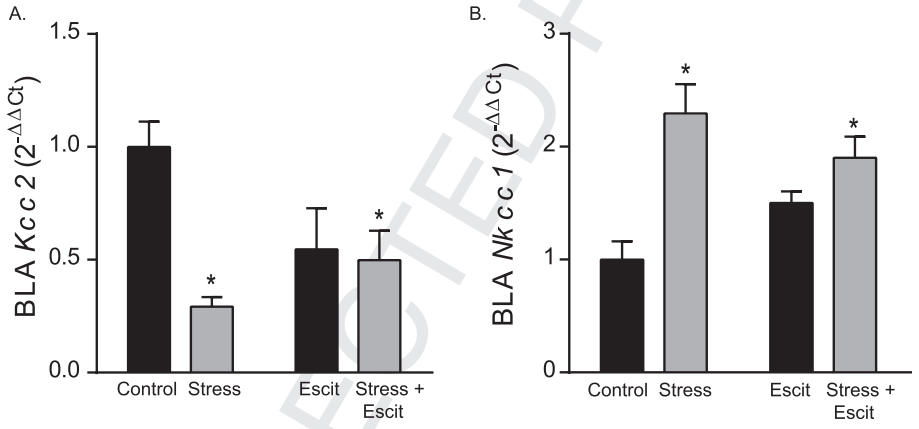

Fig. 4. Effects of prenatal stress and escitalopram on gene expression of chloride transporters in the BLA of adolescent female offspring. *A*) Prenatal stress exposure decreased gene expression of *Kcc2*, normalized to adolescent Controls. This effect was not mitigated by escitalopram. *B*) Prenatal exposure to stress or the combination of stress and escitalopram increased gene expression of *Nkcc1*, normalized to Controls, in the BLA. For all, * indicates $p < 0.05$ and error bars indicate standard error of the mean (S.E.M.).

Table 1
Effects of prenatal stress and escitalopram on gene expression of chloride transporters and serotonin receptors in the BLA of adult female offspring.

		Kcc2	Nkcc1	5-Ht_{1a}	5-Ht_{7}
Saline	No Stress	1 ± 0.227	1 ± 0.248	1 ± 0.240	1 ± 0.235
	Stress	0.810 ± 0.186	1.011 ± 0.236	0.771 ± 0.223	0.472 ± 0.122
Escitalopram	No Stress	0.870 ± 0.273	1.209 ± 0.403	0.752 ± 0.318	0.847 ± 0.281
	Stress	0.960 ± 0.276	1.002 ± 0.301	1.173 ± 0.312	0.954 ± 0.192

Values expressed are mean fold change ($2^{-\Delta\Delta Ct}$) ± SEM.

3.5. Prenatal escitalopram increases expression of inhibitory 5-Ht_{1a} in adolescent offspring amygdala

We next tested the effects of prenatal stress and escitalopram on serotonin receptor expression in offspring BLA (Fig. 5A). Exposure to prenatal escitalopram, but not stress, led to a pronounced, approximately 12-fold increase during adolescence in mRNA expression for the inhibitory, G_i-coupled serotonin receptor, 5-Ht_{1a} (Fig. 5A; $F_{1,28} = 5.89$, $p < 0.05$; n = 8). There was no effect of prenatal stress ($p > 0.05$) nor an interaction of prenatal stress and escitalopram on expression of 5-Ht_{1a} ($p > 0.05$). In contrast to the effect on 5-Ht_{1a}, there was no effect of prenatal stress or escitalopram on BLA expression of the excitatory, G_s-coupled serotonin receptor, 5-Ht_7 (Fig. 5B; p > 0.05 in all cases).

Similar to the observed changes to chloride transporter expression, by adulthood all effects on 5-Ht_{1a} expression in adolescence had abated. No effects of prenatal stress, escitalopram, or the combination of the two were observed on 5-Ht_{1a} in the BLA of adult offspring (Table 1; $p > 0.05$ in all cases). Similarly, no significant effects of any treatment were observed on adult BLA expression of 5-Ht_7 (Table 1; $p > 0.05$ in all cases).

3.6. Neither prenatal stress nor escitalopram altered serum corticosterone or estradiol in adolescence

In contrast to gene expression and behavior, physiological measures of the stress response were not altered by prenatal stress or escitalopram (Supplemental Fig. S3). Serum corticosterone

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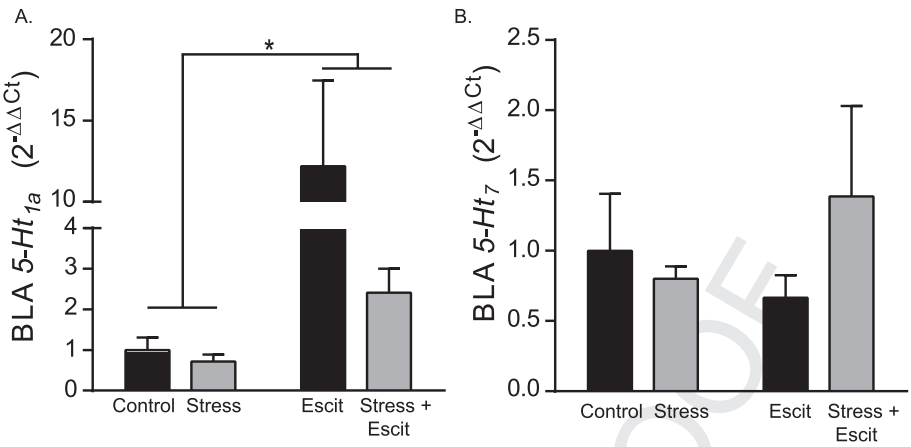

Fig. 5. Effects of prenatal stress and escitalopram on gene expression of serotonin receptors in the BLA of adolescent female offspring. *A)* Gene expression, normalized to Controls, of $5Ht_{1a}$ in the BLA of adolescent rats was increased by prenatal exposure to escitalopram. *B)* Expression of $5HT_7$ was not altered by either prenatal stress or prenatal escitalopram. For all, * indicates $p < 0.05$ and error bars indicate standard error of the mean (S.E.M.).

concentrations, measured after a 30 min restraint stressor in a separate cohort of animals, were not altered by prenatal exposure to either stress or escitalopram. Control (432.30 ± 57.76 ng/mL), Stress (427.61 ± 32.73 ng/mL), Escit (404.03 ± 42.87 ng/mL), and Stress + Escit (454.81 ± 22.51 ng/mL) groups all had comparable concentrations of serum corticosterone as measured by a two-way ANOVA ($p > 0.05$ in all cases). Similarly, estradiol concentrations did not differ by group (Control: 33.10 ± 1.88 pg/mL; Stress: 28.30 ± 2.27 pg/mL; Escit: 29.28 ± 1.0 pg/mL; and Stress + Escit: 30.88 ± 1.21 pg/mL; $p > 0.05$).

4. Discussion

Collectively, these data demonstrate that prenatal exposure to chronic, unpredictable mild stress influenced behavior of female offspring and their expression of genes regulating neurotransmission in the BLA. Our behavioral studies have shown that prenatal escitalopram did not mitigate the anxiogenic effect of prenatal stress in adolescent female offspring. Similarly, escitalopram did not mitigate the adverse impact of prenatal stress on memory in adulthood. We followed up these findings by investigating changes in neurotransmitter-related gene expression in the amygdala that could contribute to the behavioral alterations caused by prenatal stress and escitalopram. Exposure to prenatal stress shifted expression during adolescence away from the mature chloride transporter, Kcc2, and towards the immature chloride transporter, Nkcc1. This change in chloride transporter expression may disinhibit the BLA, potentially accounting for the anxiogenic effects observed in adolescence.

4.1. Behavioral effects of prenatal stress and escitalopram

Behavioral effects of prenatal stress and sex differences within the manifestation of these effects are prevalent across stress models and strains of rats (Mueller and Bale, 2007; Tibu et al., 2014; Veru et al., 2014); however, prenatal chronic stress in conjunction with *in utero* antidepressant exposure is less well understood. In our hands, prenatal stress increased anxiety-like behavior in adolescent female offspring. Prenatal escitalopram did not mitigate the effects of prenatal stress because females exposed to both manipulations exhibited altered central tendency in the open field and elevated risk-assessment behavior in the EPM. The demonstration of prenatal stress-induced increases in anxiety-like behavior is consistent with a precedent for anxiogenic effects of prenatal stress in adolescent female offspring (Baker et al., 2008) and chronic stress during other phases of development has also been shown to produce longstanding changes in female anxiety-like behaviors (Bourke and Neigh, 2011).

All adolescent females, regardless of prenatal treatment or lack thereof, exhibited deficits in novel object recognition. However, prenatal stress prevented the typical emergence of this capacity in adulthood. There is a precedent for deficits in novel object recognition in adolescence, and studies of prenatal stress have found no effects on object recognition at this age (Markham et al., 2010). Our results confirm previous findings that prenatal stress exposure alters cognitive performance in adult rodents (Abdul Aziz et al., 2012; Markham et al., 2010; Schulz et al., 2011). These data suggest that prenatal exposure to chronic stress has long-lasting, detrimental effects to the offspring that are not mitigated by escitalopram. The behavioral effects of prenatal stress were observed despite no change in serum corticosterone concentrations, suggesting that organizational changes to neural signaling are triggered early in development which persist and mature through adolescence and into adulthood.

4.2. Prenatal stress shifts expression of chloride transporters to a more excitatory configuration in the adolescent BLA

Altered chloride transporter expression has been observed following neuronal stress (Wake et al., 2007), acute stress in rodents (Maguire, 2014; Sarkar et al., 2011), and seizures (Toole et al., 2014), suggesting GABA physiology is susceptible to environmental influence. We provide evidence that prenatal stress shifts mRNA expression in the BLA away from *Kcc2* and towards *Nkcc1*. By adulthood, we observed the mature configuration of chloride transporter expression, regardless of prenatal condition, suggesting prenatal stress may delay until adolescence the typical developmental switch in transporter expression (Ehrlich et al., 2013).

Around two weeks of age in the rat BLA, expression of the chloride accumulator, *Nkcc1*, typically declines and that of the chloride extruder, *Kcc2*, emerges, driving a reduction in intracellular chloride levels and a switch from excitatory to inhibitory $GABA_A$ receptors (Ehrlich et al., 2013). These receptors mediate all of the fast synaptic inhibition in the adult BLA and their function is classically enhanced by anxiolytic drugs including benzodiazepines and barbiturates (Ehrlich et al., 2009; Rainnie et al., 1991). Therefore, the reduction in the ratio of *Kcc2:Nkcc1* should decrease

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inhibitory tone in the amygdala by rendering $GABA_A$ receptors less inhibitory.

Diminished amygdala inhibition has been argued to drive anxiety disorders in humans (Quirk and Gehlert, 2003; Rainnie et al., 2004), and we propose the anxiogenic effect of prenatal stress is due to a reduction in relative expression of *Kcc2* to *Nkcc1* in the BLA of adolescent females. Furthermore, altered chloride transporter expression in the developing amygdala may confer susceptibility for developmental psychiatric disorders, and a similar shift towards *Nkcc1* expression was recently implicated in autism spectrum disorders (Tyzio et al., 2014).

4.3. Prenatal escitalopram increases 5-Ht_{1a} expression in the adolescent BLA and alters social behavior

Gene expression of *5-Ht$_{1a}$* was increased in the adolescent BLA more than 10-fold following *in utero* exposure to chronic escitalopram. Alterations to 5-HT systems following prenatal exposure to antidepressants have been reported previously (Cabrera-Vera and Battaglia, 1998; Bourke et al., 2014). In the current study, the effect of escitalopram was specific to the inhibitory, G_i-coupled 5-Ht_{1a} receptor, as no effect of prenatal escitalopram was observed on expression of the excitatory, G_s-coupled *5-Ht$_7$*. Previous studies have reported decreased expression of *5Ht$_{1a}$* in the brain following early life stress (Franklin et al., 2011) and unpredictable stress in adulthood (Hazra et al., 2012), but neither of these previous studies nor the present study have identified an effect on serotonergic function due to prenatal stress (Abdul Aziz et al., 2012). 5-HT_{1a} receptors can influence amygdala function via action at autoreceptors (Fisher et al., 2006), but the observed effects on mRNA expression in the BLA likely reflect postsynaptic changes that should directly regulate amygdala neuron excitability rather than local serotonin release. Interestingly, females typically express higher postsynaptic 5-HT_{1A} binding than males (Schiller et al., 2006), so effects of prenatal escitalopram on BLA receptor expression and emotional behavior may be sex-specific. The effects of escitalopram exposure on gene expression in adolescence were not observed in adulthood, and we previously reported a comparable lack of effects for adult male offspring exposed to identical procedures *in utero* (Bourke et al., 2013b).

While prenatal exposure to antidepressants has been investigated for possible links to cardiovascular malformations (Malm, 2012), hypertension (Grigoriadis et al., 2014), and autism spectrum disorders (Sørensen et al., 2013), little risk has been documented (Bourke et al., 2014). The changes reported here in terms of serotonin receptor expression are transient as they were not noted in adulthood. We also report a minor change in social behavior for offspring that had been exposed to escitalopram *in utero* (Fig. 2A), but this attenuation in social behavior is transient (Fig. 2B) and does not appear to be coupled to deficits in the other behavioral endpoints examined (Figs. 1, S1, S2, 3).

Together, these data suggest that prenatal stress has both significant and long-lasting effects on neurodevelopment and behavior of female offspring. Effects of prenatal chronic stress on behavior, including emotional behavior and learning and memory, were observed regardless of concurrent prenatal exposure to escitalopram. These findings suggest prenatal stress causes transient, age-dependent deficits in anxiety-like behavior and amygdala function in female offspring, regardless of antidepressant exposure.

Uncited reference

Q8 Parks et al., 1998.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2015.05.012.

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