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Lower Anxiogenic Effects of Serotonin Agonists are Associated with Lower Activation of Amygdala and Lateral Orbital Cortex in Adolescent Male Rats

Andrew E. Arrant¹, Elizabeth Coburn¹, Jacob Jacobsen², and Cynthia M. Kuhn¹

¹Department of Pharmacology & Cancer Biology, Duke University Medical Center, Durham, NC, 27710, USA

²Department of Cell Biology, Duke University Medical Center, Durham, NC, 27710, USA

Abstract

There has been controversy over use of selective serotonin reuptake inhibitors (SSRIs) to treat affective disorders in children and adolescents due to clinical reports of increased risk for suicidal ideation and behavior during treatment, and animal studies showing changes in adult anxiety- and depressive-like behaviors after repeated treatment during adolescence. However, the acute effect of serotonergic drugs on affective behavior during adolescence is poorly understood. We investigated serotonergic modulation of anxiety-like behavior in adolescent (PN28-32) and adult (PN 67-73) male rats using the SSRI fluoxetine, the 5-HT_{1A} agonist 8-OH DPAT, and the 5-HT₂ agonist mCPP. Acute treatment with fluoxetine (10 mg/kg, i.p.) produced greater anxiogenic effects in adults than adolescents in the light/dark (LD) test for anxiety-like behavior, but fluoxetine (2.5, 5, and 10 mg/kg, i.p.) increased extracellular serotonin in the medial prefrontal cortex similarly in both ages. Adults were also more sensitive to the anxiogenic effects of 8-OH DPAT (0.25 and 0.5 mg/kg, i.p.), but not mCPP (0.5 and 1 mg/kg, i.p.), in the LD test. Fluoxetine (10 mg/kg) stimulated greater increases in c-Fos expression across the extended amygdala in adults than in adolescents, and 8-OH DPAT (0.5 mg/kg) produced greater increases in c-Fos in the lateral orbital cortex and central nucleus of the amygdala in adults. These data show that lower anxiogenic effects of acute SSRIs in adolescents are associated with lesser activation of cortical and amygdala brain regions. This immaturity could contribute to the different profile of behavioral effects observed in adolescents and adults treated with SSRIs.

Keywords

Adolescence; Serotonin; Anxiety; Microdialysis; c-Fos

1. Introduction

Adolescence is the period of transition from childhood to adulthood, and involves social, cognitive, and affective maturation (Casey et al., 2008; Ernst et al., 2006; Spear, 2000;

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Corresponding Author: Dr. Cynthia M. Kuhn Dept. of Pharmacology & Cancer Biology Duke University Room 100B Research Park Building 2 Box 3813 Duke University Medical Center Durham, NC 27710 Phone: (919) 684-8828 Fax: (919) 681-8609 ckuhn@duke.edu.

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Steinberg, 2005). Development of cortical and limbic brain regions during adolescence makes it a vulnerable period for the emergence of disorders such as depression, anxiety disorders, and drug addiction (Casey et al., 2008; Kessler et al., 2005; Steinberg, 2005). Selective serotonin reuptake inhibitors (SSRIs) are commonly used to treat affective disorders such as depression and anxiety disorders in adolescents, although SSRI treatment may initially increase suicidal ideation and behavior in children and adolescents (Barbui et al., 2009; Hammad et al., 2006). This is especially concerning given that suicide is a major cause of death in adolescents (Eaton et al., 2012). Furthermore, animal studies have reported that chronic exposure to SSRIs during adolescence may result in long term changes in adult anxiety- and depressive-like behavior (de Jong et al., 2006; Oh et al., 2009; Iniguez et al., 2010; Homberg et al., 2011).

Immaturity of neuromodulatory systems such as the central serotonergic system may represent one component of cortico-limbic maturation during adolescence that contributes to the emergence of affective disorders and drug abuse (Casey et al., 2008; Chambers et al., 2003; Ernst et al., 2006). Deficits in central serotonergic function in adults are associated with aggression, impulsivity, risk taking, and suicide (Higley and Linnoila, 1997; Mann, 2003). Currently available biochemical data indicate that the central serotonergic system continues to develop during adolescence. In rodent studies, our lab and others have shown that early adolescent rats (PN28-34) have lower forebrain tissue serotonin content and less potassium-stimulated serotonin release in the medial prefrontal cortex (mPFC) than adult rats (Arrant et al., 2013; Lidov and Molliver, 1982; Loizou, 1972). Expression of serotonin receptor subtypes appears to be generally mature by adolescence, though some studies show “pruning” of cortical 5-HT_{1A} and 5-HT_{2A} receptors between adolescence and adulthood (Li et al., 2004; Miquel et al., 1994; Slotkin et al., 2008; Xu et al., 2002). Much less information is available on the adolescent human serotonergic system. Levels of serotonin and 5-HIAA in cerebrospinal fluid are similar between adolescents and adults, although most reported studies lack statistical power to detect age-related differences (Leckman et al., 1980; Seifert et al., 1980). A single study reports that 5-HT_{1A} receptor binding in the cortex and raphe nuclei is higher in adolescents than middle aged adults (Dillon et al., 1991). These studies suggest that both serotonin-mediated behaviors and behavioral responses to serotonergic drugs may continue to mature during adolescence. However, the potential serotonergic contribution to age-specific responses to antidepressants is poorly understood.

The goal of this study was to investigate the maturity of serotonergic function and pharmacologic modulation of behavior by serotonergic drugs during adolescence. We previously found that early adolescent rats are less sensitive to the anxiogenic effects of the serotonin-releasing drug fenfluramine in unconditioned tests for anxiety-like behavior, and exhibit lower fenfluramine-stimulated serotonin release in mPFC (Arrant et al., 2013). However, we did not explore other explanations for this behavioral result. This study extends our previous findings by investigating both pre- and postsynaptic serotonergic function using the serotonin uptake inhibitor fluoxetine, the 5-HT_{1A} agonist 8-OH DPAT, and the 5-HT₂ agonist mCPP in adult and adolescent male rats. We assessed the anxiogenic effects of acute treatment with the SSRI fluoxetine in the light/dark (LD) test for anxiety, and in separate experiments used microdialysis to measure levels of extracellular serotonin in the mPFC following fluoxetine treatment. We used fluoxetine as a model reuptake inhibitor because it is the only SSRI currently approved by the FDA for treatment of pediatric depression. The contribution of 5-HT_{1A} and 5-HT₂ receptors to adolescent behavior in the LD test was assessed by treatment with 8-OH DPAT and mCPP. Finally, activation of brain regions associated with anxiety-like behavior following treatment with fluoxetine and 8-OH DPAT was measured in each age group using expression of the immediate early gene c-Fos. This study shows that lesser anxiogenic responses to serotonergic drugs extend beyond releasing drugs, and suggests that the mechanism

responsible is likely immaturity of postsynaptic neural responses to 5-HT_{1A} serotonergic signaling in the extended amygdala and/or cortex. The finding of altered anxiety responses to a clinically-used drug suggests that further study of behavioral responses to serotonergic drugs in adolescents is warranted.

2. Materials and Methods

2.1 Animals

Young adult (PN60-63) and juvenile (PN21) male Sprague-Dawley (CD) rats were purchased from Charles River Laboratories (Raleigh, NC) and arrived at our AALAC accredited facility at least 7 days before tissue or microdialysis samples were collected. The rats were housed on a 12:12 hour light/dark cycle with lights on at 06:00 and lights off at 18:00. Except as noted for microdialysis experiments, all animals were group housed with two adults per cage and three to four adolescents per cage. All experiments were approved by the Duke University Institutional Animal Care and Use Committee.

2.2 Drugs

Fluoxetine hydrochloride (Spectrum Chemicals, New Brunswick, NJ) was dissolved in distilled water, and 8-hydroxy-2-(dipropylamino)tetralin (8-OH DPAT) (Sigma Aldrich, St. Louis, MO) and meta-chlorophenylpiperazine (mCPP) (Tocris Bioscience, Bristol, UK) were dissolved in saline the morning of testing and injected i.p. at a volume of 1 mL/kg. Ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (Lloyd, Inc., Shenandoah, IA) were administered i.m. at a volume of 1 mL/kg. Ibuprofen (Children's Motrin, McNeil PPC, Fort Washington, PA) was administered via drinking water at a concentration of 0.1 mg/mL.

2.3 Light/Dark Test

Rats were tested in locomotor boxes (Kinder Scientific, Inc. Poway, CA) (40 cm × 40 cm × 40 cm) with black plastic inserts (20 cm × 40 cm × 40 cm) that occupied half of the box. Brightness of the light compartment averaged 65 lux. **Animals were tested between 8 am and 3 pm.** Rats were placed in the dark compartment and testing began as the door to the light compartment was raised. Time and distance traveled in each compartment were measured for 15 minutes with infrared photobeams and Kinder Scientific software. The latency to emerge into the light compartment was determined by dividing the session into 5 second bins and recording the bin of first light entry. Time spent in the light and latency to emerge were used as measures of anxiety-like behavior, and the total distance traveled was used to assess locomotion. Fluoxetine (10 mg/kg) was administered 30 minutes before testing, 8-OH DPAT (0.25 and 0.5 mg/kg) was administered 10 minutes before testing, and mCPP (0.5 and 1 mg/kg) was administered 20 minutes before testing. These doses and pre-treatment intervals were chosen based on prior studies of their behavioral effects in tests for anxiety-like behavior (Bodnoff et al., 1989; Critchley and Handley, 1987; Kennett et al., 1989). Behavior of drug treated animals was compared to age-matched vehicle controls that were tested at the same post-injection intervals as drug treated animals. The sample sizes for each experiment were as follows: fluoxetine n=17-19 per treatment group, 8-OH DPAT n=8 per treatment group, mCPP n=8-10 per treatment group. Treatment groups for all experiments included adult and adolescent vehicle- and drug-treated rats.

2.4 Stereotaxic Surgery

Microdialysis guide cannulae (BASi, West Lafayette, IN) were implanted over the mPFC by stereotaxic surgery under ketamine (100 mg/kg i.m.) and xylazine (20 mg/kg i.m.) anesthesia. Coordinates in mm for placement anteroposterior (AP) and mediolateral (ML) to

bregma and dorsoventral (DV) to the surface of the brain were: +3.2 AP, +0.8 ML, and -3.0 DV for adults and +2.8 AP, +0.8 ML, and -2.5 DV for adolescents. Animals recovered for at least 48 hours before microdialysis. All animals were singly housed after surgery to prevent damage to the guide cannula. Ibuprofen (0.1 mg/mL) was administered in drinking water until the night before microdialysis experiments.

2.5 Microdialysis set up and collection of baseline samples

The evening prior to all experiments, animals were briefly anesthetized with isoflurane and a 2 mm microdialysis probe (BASi) was inserted into the cannula. Animals were placed in a clear plastic chamber (BASi) and the probe was secured by a tether attached to a plastic collar. The probe was perfused overnight with artificial cerebrospinal fluid (aCSF, 147 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl₂, 0.85 mM MgCl₂, CMA Microdialysis, Solna, Sweden) at 0.5 μ L/min using a syringe pump (Harvard Apparatus, Holliston, MA). Food and water were freely available throughout the experiment. The following morning the flow rate was increased to 1 μ L/min for one hour before collection of baseline samples. Baseline samples were collected for two hours and used as an internal control for each animal. One adult and one adolescent rat were run simultaneously for each experiment.

2.6 Zero Net Flux

After collection of baseline samples, aCSF containing 0.5, 1, or 2.5 nM serotonin was perfused through the probe. After a 40 minute equilibration period, four 20 minute samples were collected. This process was repeated for all three concentrations. The concentration of serotonin perfused into the probe (5-HT_{in}) and the difference between 5-HT_{in} and serotonin in the dialysate (5-HT_{in} - 5-HT_{out}) were used to estimate *in vivo* probe recovery and extracellular serotonin concentration (Justice, 1993). The syringe contents were analyzed each day to obtain 5-HT_{in}. Seven adult and nine adolescent rats were used for this experiment.

2.7 Fluoxetine dose response

Animals were sequentially injected with 2.5, 5, and 10 mg/kg fluoxetine, doses previously shown to increase extracellular serotonin, with two hours between each dose (Rutter and Auerbach, 1993). Samples were collected at 20 minute intervals. Thirteen adult and thirteen adolescent rats were used for this experiment.

2.8 Fluoxetine infusion

Fluoxetine (30 μ M) was infused through the microdialysis probe to investigate the effects of uptake inhibition without the influence of fluoxetine metabolism or somatodendritic 5-HT_{1A} autoreceptors. The aCSF in the syringe during baseline collection was replaced with aCSF containing 30 μ M fluoxetine, a half-maximal dose for increasing extracellular serotonin in the prefrontal cortex (Hervas and Artigas, 1998). Samples were collected at 20 minute intervals for four hours during fluoxetine infusion. Ten adult and seven adolescent rats were used for this experiment.

2.9 Verification of probe placement

Brains were removed and postfixed in 10% formalin, cut into 30 μ m sections on a cryostat, and stained with cresyl violet to confirm probe placement (Fig. S1). Animals with probes placed greater than ± 0.5 mm away from the target of +3.2 mm AP were excluded from further analysis (two adults and three adolescents).

2.10 HPLC detection for microdialysis

Dialysates were injected onto a 2.1×100 mm reversed phase C18 column (Phenomenex, Torrance, CA). The mobile phase was run at 0.2 mL/min and consisted of 150 mM NaH_2PO_4 , 4.8 mM citric acid, 3 mM SDS, 50 μM EDTA (Sigma Aldrich), with 11% methanol and 17% acetonitrile (EMD Chemicals, Philadelphia, PA), pH=5.6. Serotonin was measured using an electrochemical detector set to 0.55V (BASi). The sensitivity was 1 fmol of serotonin in a 15 μL injection. Samples were quantitated with an external standard curve run each day.

2.11 ^3H -8-OH DPAT Binding

Samples of prefrontal cortex, amygdala, and hippocampus from adult and adolescent rats were dissected using a brain block, frozen on dry ice, and stored at -80°C . A single point binding analysis was performed for each sample with 1 nM ^3H -8-OH DPAT (Perkin Elmer, Waltham, MA) so that age differences in either the affinity or total number of binding sites could be detected (Xu et al., 2002). Samples were thawed and homogenized with a dounce homogenizer in 20 volumes of Tris buffer (50 mM Tris, 2 mM MgCl_2 , 2 mM Sodium Ascorbate, pH 8.0) prior to incubation (25 μg of protein per tube) with ^3H -8-OH DPAT for 1 hour at room temperature. Serotonin (400 μM) was used for determination of nonspecific binding. The reactions were terminated by the addition of 3 mL of ice cold buffer, and filtered onto glass fiber filters (Cambridge Technology, Watertown, MA) presoaked in 0.05% polyethylenimine. No age differences were detected in single point binding, so saturation binding assays were not conducted. A total of 16 rats were used for this experiment (8 per age group).

2.12 c-Fos Immunostaining

Adult and adolescent rats were treated with vehicle (saline or distilled water), 10 mg/kg fluoxetine, or 0.5 mg/kg 8-OH DPAT and transcardially perfused two hours later with phosphate buffered saline followed by 10% formalin. Injections and perfusions were performed between 8am and 2pm. Animals treated with saline and distilled water were treated as a single vehicle group for comparison with fluoxetine and 8-OH DPAT. Based on prior studies, c-Fos immunoreactive (c-Fos-IR) cells were counted in prefrontal cortex (prelimbic (PrL) and lateral orbital (LO) regions), bed nucleus of the stria terminalis (lateral (BNSTl) and ventral (BNSTv) subdivisions), amygdala (central (CeA) and medial (MeA) amygdala nuclei), and the paraventricular nucleus of the hypothalamus (PVH) (Fig. S2) (Compaan et al., 1996; Sumner et al., 2004). Brains were cut into 30 μm sections on a cryostat, and three sections per rat were stained for each brain region. The sections were incubated with an anti-c-Fos antibody (EMD Millipore, Billerica, MA) followed by a biotinylated anti-rabbit antibody (Vector Laboratories, Burlingame, CA), and developed using the ABC-NiDAB method (Vector Laboratories).

Imaging and quantitation were performed blinded to age and treatment. Sections were imaged using a Zeiss Axioimager microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) and color camera (QImaging, Inc. Surrey, British Columbia, Canada) with Metamorph software (Molecular Devices, LLC, Sunnyvale, CA). A 20X image was taken of the region of interest from both hemispheres. Digital shading correction was applied during image acquisition using Metamorph. Images were thresholded using ImageJ software prior to counting c-Fos-IR cells. Cells were thresholded based on the lowest threshold that could be set without including background pixels. The number of c-Fos-IR cells and the percent increase in c-Fos-IR cells relative to the age-matched vehicle group were used to assess drug responses. The percent increase in c-Fos was used due to age differences in c-Fos expression in vehicle-treated rats. Twelve animals per treatment group (adult and adolescent vehicle, fluoxetine, and 8-OH DPAT) were used for this experiment.

2.13 Statistics

LD data were analyzed by ANOVA with age and treatment as factors using NCSS 2004 (NCSS, Kayesville, UT). Both fluoxetine microdialysis experiments were analyzed by repeated measures ANOVA using NCSS 2004. The dose response experiment was analyzed with factors of age, time, and dose, and the infusion experiment was analyzed with factors of age and time. Data from the fluoxetine dose response experiment were log transformed prior to analysis to normalize the variance across doses. Zero net flux results were analyzed by t-test using Graphpad Prism 5.0 (Graphpad, La Jolla, CA). ^3H -8-OH DPAT binding data were analyzed by two way repeated measures ANOVA with age and brain region as factors using NCSS 2004. The number of c-Fos-IR cells and percent increase in cells relative to vehicle were analyzed by three way repeated measures ANOVA with age, treatment, and region as factors using NCSS 2004. The global cell count analysis was followed by two-way ANOVA for each brain region with age and treatment as factors. The percent increase analysis only included drug-treated animals, and was followed by ANOVA with age and region as factors for each drug. Brain regions were grouped for these analyses into extended amygdala (CeA, MeA, BNSTl, and BNSTv), prefrontal cortex (PrL and LO), and PVH based on known associations in mediating anxiety-like behavior. In analysis of all experiments, significant three-way interactions were followed by lower-order two-way ANOVAs and significant two-way interactions were followed by Newman-Keuls post-hoc testing with significance set at $p < 0.05$.

3. Results

3.1 Fluoxetine Light/Dark Test

Fluoxetine (10 mg/kg) produced greater anxiogenic effects in adult rats than in adolescents in the LD test. Adults spent less time in the light compartment than adolescents (Fig. 1a) [main effect of age $F(1,68)=30.03$, $p < 0.001$], and took longer to emerge into the light (Fig. 1b) [main effect of age $F(1,68)=27.74$, $p < 0.001$]. Fluoxetine increased latency to emerge in adult rats but not adolescents [age $\times$ treatment interaction $F(1,68)=8.78$, $p < 0.01$], indicating a greater anxiogenic effect in adults. Fluoxetine suppressed distance traveled similarly in each age group (Fig. 1c) [main effect of treatment $F(1,68)=15.48$, $p < 0.001$]. Adult rats traveled less distance than adolescents [main effect of age $F(1,68)=6.26$, $p < 0.05$], but fluoxetine did not produce significantly different effects on distance traveled between age groups.

3.2 Fluoxetine Microdialysis

Microdialysis was conducted to determine whether the greater behavioral effects of fluoxetine in adult rats were associated with greater fluoxetine-stimulated increases in extracellular serotonin. Fluoxetine (2.5, 5, and 10 mg/kg, i.p.) produced similar increases in extracellular serotonin in each age group. Each subsequent dose of fluoxetine produced greater extracellular serotonin levels (Fig. 2a) [main effect of dose $F(3,72)=327.14$, $p < 0.001$], resulting in a cumulative increase in extracellular serotonin over time [main effect of time $F(5,120)=23.85$, $p < 0.001$, time $\times$ dose interaction $F(15,334)=5.29$, $p < 0.001$]. No significant effect of age or interaction of age with other factors was detected, indicating a similar response to fluoxetine in each age group for both extracellular serotonin levels and the percent increase from baseline (Fig. 2b). Fluoxetine infusion (30 μm) also produced a similar increase in extracellular serotonin levels (Fig. 2c) [main effect of time $F(17,254)=77.79$, $p < 0.001$] and percent increase from baseline (Fig. 2d) [main effect of time $F(17,254)=43.46$, $p < 0.001$] in each age group. No significant age differences were detected in baseline extracellular serotonin levels (Fig. 2e) or *in vivo* probe recovery (Fig. 2f) using the zero net flux method of microdialysis.

3.3 8-OH DPAT Light/Dark Test

8-OH DPAT produced greater anxiogenic effects in adults compared to adolescents in the LD test. Treatment with 8-OH DPAT reduced the time spent in the light compartment (Fig. 3a) in adults relative to saline treatment, but not in adolescents [age $\times$ treatment interaction, $F(2,28)=7.81$, $p<0.01$]. 8-OH DPAT increased the latency to emerge into the light compartment (Fig.3b) [main effect of treatment $F(2,28)=4.17$, $p<0.05$], but this effect was not significantly different between age groups. However, adult rats took longer to emerge than adolescents [main effect of age $F(1,30)=9.13$, $p<0.01$]. 8-OH DPAT reduced distance traveled similarly in each age group (Fig.3c) [main effect of treatment $F(2,28)=17.24$, $p<0.001$].

3.4 mCPP Light/Dark Test

mCPP produced anxiogenic effects as shown by reduced time in the light compartment (Fig. 3d) [main effect of treatment $F(2,66)=9.56$, $p<0.001$] and increased latency to emerge (Fig. 3e) [main effect of treatment $F(2,66)=16.33$, $p<0.001$], but no age differences were detected in these effects. mCPP reduced locomotion in both age groups at 1 mg/kg (Fig.3f), though 0.5 mg/kg mCPP produced opposite trends for each age group [main effect of treatment $F(2,66)=21.9$, $p<0.001$, age $\times$ treatment interaction $F(2,66)=5.37$, $p<0.01$]. Post-hoc testing showed that mCPP comparably reduced locomotion in each age group at 1 mg/kg and that the trends at 0.5 mg/kg were not significant.

3.5 ^3H -8-OH DPAT Binding

The age differences in the anxiogenic effects of 8-OH DPAT were further investigated by ^3H -8-OH DPAT binding in homogenates of prefrontal cortex, amygdala, and hippocampus. There were regional differences in ^3H -8-OH DPAT binding [main effect of region $F(2,27)=388.22$, $P<0.001$], but no age differences were detected (Fig.4).

3.5 c-Fos Immunostaining

Fluoxetine and 8-OH DPAT produced greater percent increases in c-Fos expression in cortical and amygdala regions associated with anxiety-like behavior in adults than in adolescents. Fluoxetine produced greater activation of c-Fos expression in the adult extended amygdala (CeA, MeA, BNSTl, BNSTv), while 8-OH DPAT produced greater effects in the adult LO and CeA. Drug responses were assessed by the number of c-Fos-IR cells (Table 1, Table S1) and the percent increase in c-Fos-IR cells relative to age-matched vehicle-treated rats (Fig.5, Table S2). Fluoxetine and 8-OH DPAT increased the number of c-Fos IR cells as shown by main effects of drug treatment in global analysis [$F(2,66)=3.46$, $p<0.05$] and in regional analyses of LO [$F(2,39)=5.42$, $p<0.01$], CeA [$F(2,66)=8.72$, $p<0.001$], BNSTl [$F(2,65)=3.47$, $p<0.05$], and PVH [$F(2,60)=3.54$, $p<0.05$]. There were age differences in the number of c-Fos-IR cells in both cortical regions, as shown by a main effect of age in PrL [$F(1,45)=6.71$, $p<0.05$] and LO [$F(1,39)=4.29$, $p<0.05$]. Global analysis of the percent increase in c-Fos expression revealed an overall greater effect of fluoxetine and 8-OH DPAT in adults than in adolescents [main effect of age $F(1,44)=8.06$, $p<0.01$]. There was also an age $\times$ treatment $\times$ region interaction [$F(6,225)=3.07$, $p<0.001$], so the response to each drug was analyzed by regional groupings of extended amygdala (CeA, MeA, BNSTl, and BNSTv), prefrontal cortex (PrL and LO), and PVH. Fluoxetine increased c-Fos more in adult rats than adolescents across the extended amygdala (Fig.5a) [main effect of age $F(1,22)=5.99$, $p<0.05$], but produced similar percent increases in the PVH in each age group. 8-OH DPAT produced greater increases in c-Fos in the adult LO (Fig.5b) [main effect of age $F(1,18)=4.98$, $p<0.05$, age $\times$ region interaction $F(1,12)=42.33$, $p<0.001$] and CeA [age $\times$ region interaction $F(3,63)=2.76$, $p<0.05$] relative to adolescents.

4. Discussion

This study shows that acute treatment with the SSRI fluoxetine is less anxiogenic in the LD test in adolescent male rats compared to adults despite producing similar increases in extracellular serotonin in each age group. Adolescents were also less sensitive to the anxiogenic effects of the 5-HT_{1A} agonist 8-OH DPAT, but not to the 5-HT₂ agonist mCPP. These data indicate that serotonergic modulation of anxiety-like behavior is immature in adolescent rats, and that this age difference may be mediated through 5-HT_{1A} signaling. The lower anxiety-like responses to the SSRI fluoxetine and to 8-OH DPAT suggest that the behavioral profile elicited by serotonin-specific drugs can be quite different in adolescents than in adults, which raises the possibility that behavior during initiation of SSRI treatment could vary significantly with age of the patient.

The lesser behavioral effects of fluoxetine in adolescent rats are consistent with our previous findings with the serotonin-releasing drug fenfluramine (Arrant et al., 2013). In our previous study, fenfluramine produced greater anxiogenic effects in adult rats than in adolescents in both time spent in the light compartment and the latency to emerge into the light in the LD test (Arrant et al., 2013). In the current study, fluoxetine only produced significant age differences in the latency to emerge, though there was a trend for an age difference in time spent in the light compartment ($p=0.101$). This lack of a significant age difference was probably due to the low magnitude of effect in adult rats. The lesser effect of fluoxetine than fenfluramine on adult rats may be explained by the smaller increase in extracellular serotonin induced by the lower efficacy of serotonin uptake inhibitors than serotonin releasers at increasing extracellular serotonin (Gundlach et al., 1997). The greatest age difference in the response to both fenfluramine and fluoxetine was the latency to emerge into the light compartment, in which adults exhibited greatly increased latency, but adolescents exhibited little to no response.

Although both fenfluramine and fluoxetine produced greater anxiogenic effects in adult rats, only fenfluramine produced greater levels of extracellular serotonin in adults. Adolescent rats have lower tissue serotonin content than adults, but similar density of serotonergic innervation to the forebrain and serotonergic neuron firing rates (Arrant et al., 2013; Lanfumey and Jacobs, 1982; Lidov and Molliver, 1982; Loizou, 1972). The response to fenfluramine, but not fluoxetine, may be limited by tissue serotonin stores, so the microdialysis results with fenfluramine and fluoxetine are consistent with biochemical indices of presynaptic serotonergic function in each age group. These data lead us to question our initial conclusion of a presynaptic mechanism for the lesser anxiety-like response to indirectly acting serotonergic agents in adolescents. The present results suggest that immaturity of neural activation downstream of postsynaptic serotonin receptors is a more likely explanation for the effects of both drugs.

The present results show that the lower behavioral effects of indirect serotonin agonists in adolescents may be due to signaling downstream of 5-HT_{1A} receptors. Previous studies of rodent anxiety-like behavior have shown that the 5-HT_{1A} and 5-HT_{2C} receptors are important in mediating the anxiogenic effects of serotonin (Bagdy et al., 2001; Burghardt et al., 2007; File et al., 1996). Results from LD tests with 8-OH DPAT and mCPP indicate that 5-HT_{1A}, but not 5-HT_{2C} receptor signaling may contribute to the age differences in fluoxetine's anxiogenic effects. These anxiogenic effects are probably mediated by postsynaptic rather than presynaptic 5-HT_{1A} receptors. 8-OH DPAT infusion into hippocampus and basolateral amygdala typically increases anxiety-like behavior that can be blocked with specific 5-HT_{1A} antagonists, while stimulation of 5-HT_{1A} autoreceptors in the dorsal and median raphe produces anxiolytic effects (Engin and Treit, 2008; File et al., 1996).

The lesser behavioral effects of 8-OH DPAT in adolescents may reflect immaturity of the neural circuits modulated by 5-HT_{1A} signaling or immature downstream cellular signaling rather than immature 5-HT_{1A} receptor expression. We detected no age differences in ³H-8-OH DPAT binding across several forebrain regions, and studies of 5-HT_{1A} receptor ontogeny generally demonstrate maturation of 5-HT_{1A} expression during the juvenile period, though there is some evidence for overexpression during adolescence (Miquel et al., 1994; Slotkin et al., 2008; Xu et al., 2002). Developmental differences in downstream 5-HT_{1A} signaling events could play a role in the lower behavioral effects of 8-OH DPAT, as 8-OH DPAT has been reported as being less effective at inhibiting adenylyl cyclase in adolescents than adults (Xu et al., 2002). However, adolescent and adult rats exhibit similar hypothermic responses to 8-OH DPAT, which in rats is mediated by postsynaptic 5-HT_{1A} receptors (Bill et al., 1991; Darmani and Ahmad, 1999). This report shows that 5-HT_{1A} signaling is mature in the more caudal, hypothalamic areas which mediate this response. Many studies show ongoing development of the reciprocal connections between the prefrontal cortex and limbic brain regions during adolescence, raise the possibility that 5-HT_{1A} signaling produces less anxiety-like behavior in adolescents due to immature forebrain circuits (Casey et al., 2008; Cressman et al., 2010; Cunningham et al., 2002).

The greater activation of extended amygdala by fluoxetine and of LO and CeA by 8-OH DPAT in adults is consistent with the greater behavioral effects of these drugs in adults. Lesion or inactivation of LO and amygdala decreases anxiety-like behavior, and performance of anxiety tests increases c-Fos in these regions (Duncan et al., 1996; Engin and Treit, 2008; Hale et al., 2008; Rudebeck et al., 2007). These data may therefore highlight the LO and CeA as important regions in mediating the age differences in anxiogenic effects of serotonin agonists.

We observed generally similar behavior in vehicle-treated adult and adolescent rats. The only measure that was consistently different across experiments was the latency to emerge into the light compartment. Adolescent rats emerged into the light compartment faster than adults, as we have shown previously (Arrant et al., 2013; Schramm-Sapota et al., 2007). This may reflect lower anxiety-like behavior, or perhaps greater impulsivity in adolescents. Previous comparisons of baseline performance in anxiety tests in untreated adolescent and adult rodents have yielded inconsistent results, perhaps due to species (rats, mice) and strain (Wistar or Sprague-Dawley rats, Swiss or CD-1 mice) differences (Andrade et al., 2003; Hascoet et al., 1999; Imhof et al., 1993; Macri, 2002; Slawecki, 2005). In contrast, adolescents have been consistently reported to be less sensitive to the anxiogenic and aversive effects of both acute drug treatment and withdrawal from drugs of abuse (Doremus et al., 2003; Infurna and Spear, 1979; O'Dell et al., 2007; Schramm-Sapota et al., 2007; Schramm-Sapota et al., 2006; Schramm-Sapota et al., 2009; Shram et al., 2006). Serotonin contributes to the anxiogenic and aversive effects of drugs of abuse, so immature serotonergic function may play a role in adolescent insensitivity to the anxiogenic and aversive effects of these drugs (Ettenberg et al., 2011; Jones et al., 2010). There is a large body of literature showing that the extended amygdala mediates the aversive effects of withdrawal from drugs of abuse due to activation by neuromodulators such as corticotropin-releasing factor and norepinephrine (Koob and Volkow, 2010). The adolescent immaturity of extended amygdala activation observed in this study may therefore show similar mechanisms between acute anxiogenic drug effects and the aversive effects of withdrawal from drugs of abuse.

The specific behavioral construct reflected by lower anxiety behavior in adolescents after serotonergic drugs is not clear. Serotonin is thought to promote behavioral inhibition in opposition dopamine-mediated approach behavior, and deficits in serotonergic function are associated with disinhibition of behavior (Boureau and Dayan, 2011; Cools et al., 2011).

The present results could therefore reflect immaturity of serotonergic behavioral inhibition as well as serotonergic modulation of anxiety-like behavior (Chambers et al., 2003; Ernst et al., 2006). Immaturity of this inhibitory serotonergic influence could promote risk taking behavior during adolescence such as experimentation with drugs. However, more direct tests of adolescent risk-taking will be required to test this possibility.

The present findings may be relevant to reports of adolescent suicidality during initiation of antidepressant treatment with SSRIs. Meta-analyses indicate that children and adolescents are at greater risk than adults for suicidal ideation and behavior during SSRI treatment (Barbui et al., 2009; Dubicka et al., 2006; Hammad et al., 2006; Schneeweiss et al., 2010). This has been a topic of considerable debate, as SSRIs have antidepressant efficacy in adolescents, and untreated depression also increases risk of suicidal behavior (Correll et al., 2011; Gibbons et al., 2012; Gibbons et al., 2007). Results about the relative hazards of different SSRIs in adolescents are inconsistent but serotonin and norepinephrine reuptake inhibitors (SNRIs) such as venlafaxine may produce greater risk than SSRIs in all age groups (Barbui et al., 2009; Dubicka et al., 2006; Rubino et al., 2007; Schneeweiss et al., 2010; Whittington et al., 2004). There is evidence that the noradrenergic system is also immature during adolescence, and may be involved in adolescent suicidality during antidepressant therapy (Murrin et al., 2007; West et al., 2010). The neurochemical and postsynaptic responses to SNRIs in adolescents may therefore be an important area for future study.

The greater behavioral effects of fluoxetine in adult rats in the LD test could indicate an increase in behavioral inhibition, which would reduce impulsive behavior. Impulsivity is associated with suicidal behavior, so the fluoxetine LD data may reflect a protective effect against suicidal behavior in adults that is absent in adolescents (Hawton et al., 2012; Mann, 2003). This lack of a protective effect against suicidality may also be observed in humans. A recent meta-analysis reported similar antidepressant effects of fluoxetine in adolescents and adults, but fluoxetine reduced suicidal ideation and behavior relative to placebo only in adults (Gibbons et al., 2012). Risk of suicidality is greatest during the first two weeks of antidepressant therapy for all age groups, so age differences in the behavioral effects of subchronic fluoxetine administration will be an important area for future study (Hall, 2006; Jick et al., 2004). The present report emphasizes the importance of understanding how therapeutically-used psychoactive drugs act differently in children and adolescents, and demonstrates the value of adequate testing in animal models and in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

8-OH DPAT	8-hydroxy-2-(dipropylamino)tetralin
mCPP	meta-chlorophenyl piperazine
LD	light/dark test for anxiety
SSRI	selective serotonin reuptake inhibitor
mPFC	medial prefrontal cortex

PrL	prelimbic cortex
LO	lateral orbital cortex
CeA	central nucleus of the amygdala
MeA	medial nucleus of the amygdala
BNSTl	lateral division of the bed nucleus of the stria terminalis
BNSTv	ventral division of the bed nucleus of the stria terminalis
PVH	paraventricular nucleus of the hypothalamus

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Highlights

- Adolescents exhibited a lesser anxiogenic response to acute fluoxetine treatment
- Fluoxetine increased extracellular serotonin similarly in each age group
- Adolescents exhibited a lesser anxiogenic response to the 5-HT_{1A} agonist 8-OH DPAT
- Adolescents had a lesser amygdala and cortex c-Fos response to fluoxetine and 8-OH DPAT
- Immaturity of amygdala and cortex may contribute to lesser anxiogenic responses

Figure 1.

The effects of 10 mg/kg fluoxetine on behavior in the light/dark test. A.) Time spent in the light compartment. B.) Latency to emerge into the light compartment. * = different from all other groups ($p < 0.05$). C.) Total distance traveled. $n = 17-19$ per treatment group. Data are shown as mean $\pm$ SEM.

Figure 2.

Results from microdialysis experiments in the mPFC. A-B.) The effects of sequential doses of 2.5, 5, and 10 mg/kg fluoxetine on A.) extracellular serotonin levels and B.) the percent increase in serotonin from baseline. $n=13$ adults and 13 adolescents. Arrows indicate time points at which fluoxetine was administered. C-D.) The effects of local infusion of 30 μ M fluoxetine into mPFC on C.) extracellular serotonin levels and D.) The percent increase in extracellular serotonin relative to baseline. $n=10$ adults and 7 adolescents. E-F.) Results of zero net flux experiment. E.) Estimated baseline extracellular concentration in mPFC. $n=7$ adults and 9 adolescents. * = significant age difference, $p<0.05$ F.) Estimated *in vivo* probe recovery. All data are shown as mean $\pm$ SEM.

Figure 3.

The effects of 8-OH DPAT (A-C, $n=8$ per treatment group) and mCPP (D-F, $n=8-10$ per treatment group) on behavior in the light/dark test. A., D.) Time spent in the light compartment. B., E.) Latency to emerge into the light compartment, C., F.) Total distance traveled. * = significantly different from age matched control, $p < 0.05$. All data are shown as mean $\pm$ SEM.

Figure 4. Binding of 1nM ³H 8-OH DPAT to homogenates of prefrontal cortex (PFC), amygdala (AMG), and hippocampus (HPC) of adult and adolescent rats. n = 8 adults and 8 adolescents.

Figure 5.

The percent increase in c-Fos immunoreactive cells relative to age matched vehicle treated animals after A.) 10 mg/kg fluoxetine and B.) 0.5 mg/kg 8-OH DPAT. * = significant age difference, $p < 0.05$. Representative images of c-Fos immunostaining in central amygdala (C-F) and lateral orbital cortex (G-J), scale bars in all images are 100 μm . Representative staining from C.) adolescent vehicle, D.) adolescent fluoxetine, E.) adult vehicle, F.) adult fluoxetine in central amygdala. Representative staining from G.) adolescent vehicle, H.) adolescent 8-OH DPAT, I.) adult vehicle, J.) adult 8-OH DPAT in lateral orbital cortex. PrL=prelimbic cortex, LO=lateral orbital cortex, CeA=central nucleus of the amygdala, MeA=medial nucleus of the amygdala, BSTl=lateral subdivision of the bed nucleus of the stria terminalis, BSTv=ventral subdivision of the bed nucleus of the stria terminalis. $n=12$ per treatment group. Data are shown as mean $\pm$ SEM.

Table 1

The number of c-Fos immunoreactive cells in each age group after treatment with vehicle, fluoxetine (10 mg/kg) or 8-OH DPAT (0.5 mg/kg). PrL=prelimbic cortex, LO=lateral orbital cortex, CeA=central nucleus of the amygdala, MeA=medial nucleus of the amygdala, BSTl=lateral subdivision of the bed nucleus of the stria terminalis, BSTv=ventral subdivision of the bed nucleus of the stria terminalis. n=12 per treatment group. Data are shown as mean $\pm$ SEM.

Age	Treatment	Region						
		PrL	LO	CeA	MeA	BSTl	BSTv	PVH
Adolescent	Vehicle	52 $\pm$ 20	38 $\pm$ 21	48 $\pm$ 7	108 $\pm$ 17	44 $\pm$ 7	104 $\pm$ 23	93 $\pm$ 13
	Fluoxetine	39 $\pm$ 13	25 $\pm$ 9	104 $\pm$ 14	110 $\pm$ 15	45 $\pm$ 10	73 $\pm$ 19	218 $\pm$ 46
	8-OH DPAT	75 $\pm$ 22	51 $\pm$ 17	92 $\pm$ 23	97 $\pm$ 16	72 $\pm$ 15	90 $\pm$ 10	118 $\pm$ 24
Adult	Vehicle	102 $\pm$ 24	28 $\pm$ 13	38 $\pm$ 10	59 $\pm$ 9	43 $\pm$ 8	78 $\pm$ 18	73 $\pm$ 14
	Fluoxetine	73 $\pm$ 29	51 $\pm$ 22	127 $\pm$ 25	118 $\pm$ 20	84 $\pm$ 14	110 $\pm$ 22	198 $\pm$ 45
	8-OH DPAT	149 $\pm$ 32	145 $\pm$ 39	126 $\pm$ 27	90 $\pm$ 15	73 $\pm$ 13	62 $\pm$ 13	122 $\pm$ 23