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Adolescent Male Rats Are Less Sensitive than Adults to the Anxiogenic and Serotonin-Releasing Effects of Fenfluramine

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Abstract

Risk taking behavior increases during adolescence, which is also a critical period for the onset of drug abuse. The central serotonergic system matures during the adolescent period, and its immaturity during early adolescence may contribute to adolescent risk taking, as deficits in central serotonergic function have been associated with impulsivity, aggression, and risk taking. We investigated serotonergic modulation of behavior and presynaptic serotonergic function in adult (67–74 days old) and adolescent (28–34 days old) male rats. Fenfluramine (2 mg/kg, i.p.) produced greater anxiogenic effects in adult rats in both the light/dark and elevated plus maze tests for anxiety-like behavior, and stimulated greater increases in extracellular serotonin in the adult medial prefrontal cortex (mPFC) (1, 2.5, and 10 mg/kg, i.p.). Local infusion of 100 mM potassium chloride into the mPFC also stimulated greater serotonin efflux in adult rats. Adult rats had higher tissue serotonin content than adolescents in the prefrontal cortex, amygdala, and hippocampus, but the rate of serotonin synthesis was similar between age groups. Serotonin transporter (SERT) immunoreactivity and SERT radioligand binding were comparable between age groups in all three brain regions. These data suggest that lower tissue serotonin stores in adolescents limit fenfluramine-stimulated serotonin release and so contribute to the lesser anxiogenic effects of fenfluramine.

Keywords

Adolescence; Serotonin; Anxiety; Microdialysis

1. Introduction

Adolescence is the period of transition from childhood to adulthood (Spear, 2000). This transitional period includes the time from around 12 to 18 years of age in humans (reviewed in Spear, 2000). In rodents, adolescence encompasses postnatal days 28 to 42 (PN28–42), though adulthood is not considered to begin until around PN60 (reviewed in McCutcheon and Marinelli, 2009; Spear, 2000). Behavior changes during adolescence, with risk taking, novelty seeking, and social behavior expressed at higher levels than in childhood or adulthood (Stansfield and Kirsstein, 2006; Steinberg et al., 2008; Steinberg et al., 2009;

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reviewed in Spear, 2000). Impulsive, risk taking behavior is part of normal development, but also contributes to major causes of adolescent injury and mortality such as reckless driving, suicide, unsafe sexual behavior, and experimentation with drugs (Chen and Kandel, 1995; Eaton et al., 2010; SAMHSA, 2011; Steinberg, 2008; reviewed in Spear, 2000).

Immature function of the neural circuits that mediate goal directed behavior contributes to adolescent risk taking. The balance between systems mediating approach to rewarding stimuli and avoidance of aversive stimuli may be biased toward approach during adolescence (reviewed in Ernst and Fudge, 2009; Ernst et al., 2006). Prefrontal cortical regulation of limbic brain regions is immature, limiting the regulation of these approach and avoidance drives (reviewed in Casey et al., 2011; Ernst and Fudge, 2009; Ernst et al., 2006; Steinberg, 2010; Sturman and Moghaddam, 2011). Immaturity of dopaminergic and serotonergic function in the forebrain may also contribute to this approach/avoidance imbalance in adolescents (reviewed in Chambers et al., 2003; Crews et al., 2007; Ernst et al., 2006). Serotonin is an important mediator of behavioral inhibition in response to aversive situations, and low central serotonergic function has been associated with risk taking, impulsivity, and aggression (Brown et al., 1979; Crockett et al., 2009; Higley and Linnoila, 1997; Higley et al., 1996; Mehlman et al., 1994; Soubrie, 1986; Virkkunen et al., 1995). Serotonin also contributes to the aversive effects of some drugs of abuse, and adolescents are less sensitive to aversive effects of drugs in animal models (Ettenberg and Bernardi, 2006, 2007; Ettenberg et al., 2011; Infurna and Spear, 1979; Jones et al., 2009; Jones et al., 2010; Rocha et al., 2002; Schramm-Sapota et al., 2006; Serafine and Riley, 2010). Lower serotonergic function in adolescents could therefore contribute to increased risk taking behavior and reduce the aversive effects of drugs of abuse. These effects could factor into the increased experimentation with drugs seen during adolescence (Chen and Kandel, 1995; SAMHSA, 2011).

Animal studies suggest that forebrain serotonergic function during early adolescence may be lower than in adults, especially in the cortex. While serotonin receptor expression, dorsal raphe firing rates, and the anatomic pattern of serotonergic innervation appear to be mature by adolescence, neurochemical markers of presynaptic serotonergic function increase between adolescence and adulthood (Beique et al., 2004; Daval et al., 1987; Garcia-Alcocer et al., 2006; Lanfumey and Jacobs, 1982; Lidov and Molliver, 1982; Miquel et al., 1994; Pranzatelli and Galvan, 1994; Vizuete et al., 1997; Waeber et al., 1996; Waeber et al., 1994). Serotonin transporter (SERT) binding is lower in the cortex of early adolescent rats (PN28-35), and some studies show lower SERT binding in subcortical regions such as the amygdala and striatum (Dao et al., 2011; Galineau et al., 2004; Moll et al., 2000; Tarazi et al., 1998). Serotonin tissue content and synaptosomal uptake are also lower in the cortex and striatum of early adolescent rats compared to adults (Kirksey and Slotkin, 1979; Loizou, 1972; Loizou and Salt, 1970; Mercugliano et al., 1996). Studies during later adolescence have reported more adult-like serotonin function. Older adolescent rats (PN45-50) and adults have similar baseline and methamphetamine-stimulated extracellular serotonin in medial prefrontal cortex (mPFC) (Staiti et al., 2011). SERT binding and serotonin uptake are also mature by this age (Kirksey and Slotkin, 1979; Tarazi et al., 1998). These studies show that neurochemical markers of presynaptic serotonergic function reach adult levels during the adolescent period, and that these markers are lower than in adults during early adolescence from PN28 to PN35.

While the ontogeny of forebrain serotonergic innervation has been described in young adolescents, serotonin release in response to pharmacologic or behavioral stimuli has not been evaluated over this critical developmental window. Additionally, the ability of serotonergic drugs to influence some behaviors has been studied in adolescents of several species, but a direct comparison of serotonergic regulation of behavioral inhibition in

adolescents and adults has not been reported (Higley et al., 1996; LeMarquand et al., 1998; Mehlman et al., 1994; Zepf et al., 2008). The purpose of this study is to address this gap in our understanding of how serotonin regulates behavior during adolescence by assessing behavioral and neurochemical responses to pharmacologic challenge of the serotonergic system in adults (PN67-74) and early adolescents (PN28-34). We used the serotonin releasing drug fenfluramine to compare the ability to mobilize serotonin stores in adults and early adolescents. Fenfluramine mobilizes serotonin stores by a similar mechanism as drugs of abuse such as methamphetamine and methylenedioxymethamphetamine (MDMA), but is more selective for the serotonergic system than these drugs (Rothman et al., 2001; reviewed in Sulzer et al., 2005). The behavioral effects of fenfluramine treatment were evaluated in the light/dark (LD) and the elevated plus maze (EPM) tests for anxiety-like behavior. Unconditioned tests for anxiety-like behavior such as the LD and EPM are thought to be an ethologically relevant model of risk taking behavior because they measure inhibition of species-typical behaviors in novel, aversive, and potentially risky environments (Harro, 2002; Macri, 2002; Olausson et al., 1999). We then used microdialysis to assess the effect of fenfluramine and potassium on extracellular serotonin in the mPFC, and further investigated presynaptic serotonin function by measuring serotonin content, synthesis, innervation density, and SERT levels in the prefrontal cortex, amygdala, and hippocampus.

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). The rats were housed in ventilated plastic cages (Techniplast USA, Exton, PA) or standard rat cages (Allentown Caging, Allentown, NJ) with corn cob bedding on a 12:12 hour light/dark cycle with lights on at 06:00 and lights off at 18:00. All rats were allowed to acclimate to our AALAC accredited facility for at least 7 days before behavior testing or collection of tissue or microdialysis samples. The acclimation period did not exceed 13 days to ensure that adolescent rats were tested during the first week of adolescence (PN28-34). The timeline from arrival in our animal facility to behavior or microdialysis testing is depicted in Figure 1. Separate cohorts of animals were used for all behavioral and microdialysis experiments to avoid potential confounding effects of repeated testing upon behavior or extracellular serotonin levels. All experiments were approved by the Duke University Institutional Animal Care and Use Committee.

2.2 Drugs

Fenfluramine hydrochloride, 3-hydroxybenzylhydrazine dihydrochloride (NSD-1015), and potassium chloride were purchased from Sigma-Aldrich (St. Louis, MO). Fenfluramine and NSD-1015 were dissolved in saline the morning of testing and injected i.p. at a volume of 1 mL/kg and 2 mL/kg, respectively. Ketamine (Ketaset) was purchased from Fort Dodge Animal Health (Fort Dodge, IA) and xylazine (Anased) was purchased from Lloyd Inc. (Shenandoah, IA). Ketamine and xylazine were administered i.m. at a volume of 1 mL/kg.

2.3 Light/Dark Test

Rats were tested in Kinder 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 locomotor box. Each insert had a sliding door that restricted access to one part of the box. The room was lit by two incandescent lamps so that the lighting in the light side of each box was 65 lux on average. Rats were placed in the dark half of the box and testing began as the door to the light side of the box was raised. Time and distance traveled in each compartment were measured for 15 minutes with infrared photobeams and software from the

manufacturer. The latency to emerge into the light compartment was determined by dividing the session into 5 second bins and determining the bin of first light entry. Adult and adolescent rats were injected with either saline or fenfluramine (2 mg/kg) 30 minutes prior to testing (n=12 per age, per treatment). This dose of fenfluramine was expected to be non-maximal in terms of increasing extracellular serotonin in the brain (Gundlah et al., 1997; Rocher and Gardier, 2001; Tao et al., 2002). For each age group, saline-treated animals were used as a control group to determine baseline behavior. Behavior of fenfluramine-treated animals was compared to age-matched saline controls for determination of drug effects. A total of 48 rats were used for this experiment (12 per experimental group, adult and adolescent rats treated with saline or fenfluramine).

2.4 Elevated Plus Maze

The elevated plus maze was made of black painted wood and consisted of two open arms and two closed arms arranged in a “plus” shape elevated 60 cm from the floor. The arms were 60 cm × 9 cm. The closed arms were enclosed by a 50 cm wall and the open arms had a 1 cm ledge. Rats were placed in an open field for 5 minutes immediately prior to testing to enhance the ability to measure anxiogenic drug effects (Pellow and File, 1986). Rats were placed in the center square (9 × 9 cm) facing an open arm and allowed to explore the maze for 5 minutes. White sheets were hung around the maze to create a neutral visual field. Average lighting was 8 lux for open arms and 2 lux for closed arms. All sessions were recorded with a video camera and scored for time in each area of the maze, entries into arms, and the latency to first open arm entry. Adult and adolescent rats were injected with either saline or fenfluramine (2 mg/kg) 30 minutes prior to placement in the open field. Behavior of fenfluramine-treated animals was compared to age-matched saline controls for determination of drug effects. A total of 60 rats were used for this experiment (15 per experimental group, adult and adolescent rats treated with saline or fenfluramine).

2.5 Stereotaxic Surgery

Animals were anesthetized with ketamine (100 mg/kg i.m.) and xylazine (20 mg/kg i.m.) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). Body temperature was maintained with a Deltaphase isothermal heating pad (Braintree Scientific, Braintree, MA). Guide cannulae (BAS, inc. West Lafayette, IN) were implanted 2 mm over the medial prefrontal cortex, and placed at +3.2 mm anterior and +0.8 mm lateral from bregma and −3.0 mm from the dura based on a rat brain atlas (Paxinos and Watson, 1986). Coordinates for adolescent rats were adjusted to +2.8 mm anterior and +0.8 mm lateral from bregma and −2.5 mm from the dura. Adolescent coordinates were empirically determined in preliminary dye injection and microdialysis experiments. The cannula was secured using three anchor screws and dental cement. Animals were allowed to recover for a minimum of 48 hours before insertion of the microdialysis probe. Ibuprofen (20 mg/kg/day) was administered through the drinking water from 3 days prior to surgery until the night before microdialysis experiments in accordance with Duke University IACUC standards.

2.6 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 (BAS, Inc. West Lafayette, IN) was inserted into the cannula. Animals were placed in a clear plastic microdialysis chamber (BAS, Inc. West Lafayette, IN) and 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 µL/min using a syringe pump (Harvard Apparatus, Holliston, MA). The microdialysis cages were bowl-shaped and had a diameter of approximately 36 cm at the bottom of the cage, and a depth of approximately 30 cm with bedding. The same size cages were used for each age group. The inlet and outlet lines for the microdialysis probe were run through a

liquid swivel (BAS, Inc, West Lafayette, IN), and animals were tethered to the swivel by plastic collars. Food and water were freely available throughout the overnight equilibration and subsequent experiments. The following morning the flow rate was increased to 1 $\mu\text{L}/\text{min}$ for one hour before collection of baseline samples. Baseline samples were then collected for 2 hours prior to experimental manipulations. Samples were collected on ice, then immediately frozen on dry ice for later analysis by HPLC with electrochemical detection. One adult and one adolescent rat were run simultaneously each day.

2.7 Fenfluramine dose response

Animals were sequentially injected with 1, 2.5, and 10 mg/kg fenfluramine i.p. with three hours between each dose. Samples were collected at 30 minute intervals. All three doses were given to the same animal on the same day. Collection of baseline samples began at 7 AM and the experiments concluded around 6 PM to ensure that all samples were collected during the light phase. The baseline samples from each animal were used as an internal control for comparison with fenfluramine-stimulated extracellular serotonin levels. A total of 17 animals were used for this experiment (9 adults and 8 adolescents).

2.8 Potassium depolarization

After collection of baseline samples, the aCSF in the syringes was replaced with aCSF containing 100 mM potassium chloride. The high potassium aCSF was perfused for 20 minutes before the syringes were switched back to normal aCSF. Samples were collected for another 4 hours with normal aCSF. All samples were collected in 20 minute intervals. A total of 22 animals were used for this experiment (12 adults and 10 adolescents).

2.9 Verification of probe placement

After conclusion of microdialysis experiments, animals were anesthetized with isoflurane and decapitated. Brains were postfixed in 10% formalin, cut into 30 μm sections, and stained with cresyl violet to confirm probe placement (Supplementary Fig. 1). Animals with probes placed greater than ± 0.5 mm anterior or posterior from the target region of 3.2 mm anterior from bregma were excluded from further analysis (1 adult and 3 adolescents). These animals are not included in the sample sizes given for the fenfluramine or potassium microdialysis experiments.

2.10 HPLC detection for microdialysis

Dialysates were directly injected onto a 2.1×100 mm reversed phase C18 column (Phenomenex, Torrance, CA). The mobile phase consisted of 150 mM sodium phosphate, 4.8 mM citric acid, 3 mM sodium dodecyl sulfate, 50 μM EDTA, with 11% methanol and 17% acetonitrile, pH=5.6 and was run at a flow rate of 0.2 mL/min. Serotonin was measured using an electrochemical detector set to 0.55V (BAS, Inc. West Lafayette, IN). The sensitivity of the assay was 1 fmol of serotonin in a 15 μL injection. Samples were quantitated relative to an external standard curve run each day.

2.11 Serotonin Synthesis and Tissue Serotonin Content

Adult and adolescent rats (n=8 per age, per treatment) were injected with saline or 100 mg/kg NSD-1015 to inhibit L-amino acid decarboxylase and decapitated 30 minutes later. Brains were collected at ages PN28-29 for adolescent rats and PN67-71 for adult rats. Levels of 5-hydroxytryptophan (5-HTP) after treatment with NSD-1015 were used to compare the rate of serotonin synthesis (Carlsson et al., 1972). Serotonin and 5-HIAA were also compared in saline treated adults and adolescents. Prefrontal cortex, amygdala, and hippocampus were dissected using a brain block (Heffner et al., 1980). The samples were weighed, frozen on dry ice, and stored at -80°C until HPLC analysis. Tissue samples were

homogenized by sonication in 0.2N perchloric acid and injected onto a 4.6×100 mm reversed phase C18 column (Phenomenex, Torrance, CA). Serotonin, 5-HIAA, dopamine, DOPAC, HVA, and 5-HTP were measured using electrochemical detection at 0.70V (BAS, West Lafayette, IN) at a flow rate of 0.7 mL/min. The mobile phase consisted of 0.1 M sodium phosphate, 0.8 mM octanesulfonic acid, 0.1 mM EDTA, and 18% methanol adjusted to pH 3.10 (Miguez et al., 1995). Samples were quantitated relative to an external standard curve run each day. Protein content per tissue wet weight is similar in the forebrain of early adolescent (PN30) and adult rats, so data from each sample were corrected for tissue weight (Porcher and Heller, 1972).

2.12 Serotonin Transporter Immunostaining

Adult (PN67-70) and adolescent (PN28) rats were anesthetized and transcardially perfused with PBS followed by 10% formalin. Brains were postfixed in 10% formalin overnight at 4°C, then cryoprotected in 30% sucrose until sectioning. The brains were cut into 30 μ m sections and mounted onto slides prior to immunostaining. After heat mediated antigen retrieval in citric acid buffer (10 mM citrate, pH 6.0), the sections were stained with an anti-SERT primary antibody (Immunostar, Hudson, WI) followed by an anti-rabbit secondary antibody conjugated to AlexFluor 594 (Invitrogen, Carlsbad, CA) and counterstained with DAPI (Invitrogen). The sections were imaged on a widefield fluorescent microscope with a motorized stage (DeltaVision Elite, Applied Precision, Issaquah, WA). Panels of images were taken of the prefrontal cortex, amygdala, and dorsal hippocampus using a 20X objective, with 7 z-stacks per panel. The images were deconvoluted, projections were made of the z-stacks, and the panels were stitched together to create a single high-resolution image. For analysis, the amygdala was subdivided into central and basolateral amygdala, and the hippocampus was subdivided into CA1, CA3, and dentate gyrus. ImageJ software was used for analysis. The images were thresholded to exclude background, and the percent of the total image area occupied by thresholded structures was used to quantitate the density of SERT-immunoreactive innervation. A total of 24 rats were used for this experiment (12 per age group).

2.13 Serotonin Transporter Radioligand Binding

Samples of prefrontal cortex, amygdala, and hippocampus were collected from adult (PN67-70) and adolescent (PN28) rats using the same procedure as for HPLC analysis. The samples were immediately frozen on dry ice and stored at -80°C until analysis. Samples were thawed and homogenized by sonication in 20 volumes of phosphate buffer (8 mM sodium phosphate dibasic, 2 mM sodium phosphate monobasic, 118 mM NaCl, 5 mM KCl, pH 7.4). The samples (10 μ g of protein per tube) were incubated with ^3H -paroxetine (Perkin Elmer, Waltham, MA) for 90 minutes at room temperature. Fluoxetine (10 μM) was used for determination of nonspecific binding. The reactions were terminated by the addition of 3 mL of ice cold buffer, and then filtered onto glass fiber filters (Cambridge Technology, Watertown, MA) presoaked in 0.05% polyethylenimine to reduce nonspecific binding.

A single point binding analysis was performed for each sample and saturation binding analysis was run on pooled adult and adolescent samples from each brain region. For single point analysis, samples were incubated with 50 pM ^3H -paroxetine so that age differences in either the affinity or total number of binding sites could be detected. The samples were then pooled for saturation analysis, with one pool per age group for each region. Pooled samples were run with a range of ^3H -paroxetine concentrations from 10 pM to 2000 pM. Nonlinear fits of saturation binding curves were performed with GraphPad Prism 5.0 (Graphpad, La Jolla, CA). Sixteen rats were used for this experiment (8 per age group).

2.14 Data Analysis and Statistics

The effects of age and fenfluramine treatment on anxiety-like behavior in the LD and EPM tests were analyzed by two-way ANOVA with Age and Treatment as factors using NCSS 2004 software (NCSS, Kayesville, UT). Data from microdialysis experiments were analyzed by repeated measures ANOVA with Age as a between factor and Time and Dose (fenfluramine only) as within factors. Repeated measures ANOVA with Age as a between factor and Brain Region as a within factor was used to investigate the effect of age on tissue serotonin and 5-HIAA content, SERT immunostaining, and ^3H -paroxetine binding across brain regions. The effects of age and NSD-1015 treatment on serotonin synthesis in multiple brain regions were analyzed by repeated measures ANOVA with Age and Treatment as between factors, and Brain Region as a within factor. For all analyses, significant three-way interactions were further investigated by lower-order two-way ANOVA and significant two-way interactions were followed by Newman-Keuls post-hoc testing with significance set at $p < 0.05$. All data are shown as mean $\pm$ SEM.

3. Results

3.1 Light/Dark Test

The time spent in the light compartment and the latency to emerge into the light were used as measures of anxiety-like behavior, and the total distance traveled was used to assess locomotion (Morley et al., 2005; Schramm-Sapota et al., 2007). Fenfluramine increased anxiety-like behavior in both ages ($n=12$ per experimental group) as shown by a reduction in time spent in the light compartment (Fig. 2a) [main effect of Treatment, $F(1,39)=77.92$, $p < 0.001$]. This effect was significantly greater in adult rats, as indicated by a significant Age X Treatment interaction [$F(1,39)=7.75$, $p < 0.01$]. Post-hoc testing showed that fenfluramine reduced the time spent in the light compartment more in adults than in adolescents. Fenfluramine produced similar age-dependent anxiogenic effects on latency to emerge (Fig. 2b) [Age X Treatment interaction $F(1,40)=5.91$, $p < 0.05$]. Post-hoc testing confirmed that fenfluramine increased latency to emerge in adult, but not adolescent, rats relative to controls. Fenfluramine reduced the number of entries into the light compartment (Fig. 2c) [main effect of Treatment $F(1,39)=28.83$, $p < 0.001$] and total distance (Fig. 2d) [main effect of Treatment $F(1,39)=50.30$, $p < 0.001$] similarly in both ages.

3.2 Elevated Plus Maze

Fenfluramine was also more anxiogenic in adult rats than adolescents in the EPM ($n=15$ per experimental group). The percent time spent in the open and closed arms of the maze, the number of entries into the open arms, and the latency to emerge into an open arm were used as measures of anxiety-like behavior, while the number of entries into closed arms was used as an index of locomotion (Cruz et al., 1994; File et al., 2004; File et al., 1993; Rogerio and Takahashi, 1992). Fenfluramine produced significantly greater anxiogenic effects in adult rats than adolescents on percent time spent in the closed arms (Fig. 3d) [Age X Treatment interaction [$F(1,55)=4.41$, $p < 0.05$], and the latency to enter an open arm (Fig. 3c) [Age X Treatment interaction $F(1,55)=4.40$, $p < 0.05$]. Post-hoc testing showed that fenfluramine-treated adults spent more time in the closed arms and took longer to enter an open arm than adult controls, while adolescents were unaffected. A main effect of Age [$F(1,55)=9.89$, $p < 0.01$] was found for the number of entries into the open arms (Fig. 3b), which was driven by a decrease in open entries in fenfluramine-treated adults. No main effects or interactions were identified by an ANOVA of percent time spent in the open arms (Fig. 3a). Several of the adult saline-treated rats had low time in open arms and open entries, which could have caused a floor effect for seeing significant treatment effects in these measures. Rat behavior in the EPM is variable and is affected by many environmental factors (File et al., 2004). Adolescents are less sensitive than adults to environmental effects on EPM behavior, which

may have contributed to the lower variability in saline-treated adolescents (Doremus et al., 2004). There were no significant effects of fenfluramine on the number of closed entries (Fig. 3e), but ANOVA revealed a main effect of Age [$F(1,55)=18.81$, $p<0.001$].

3.3 Fenfluramine Microdialysis

Fenfluramine-stimulated serotonin levels were greater in adult rats ($n=9$) than adolescents ($n=8$). The baseline serotonin levels were used as a within-animal control for comparison with fenfluramine-stimulated extracellular serotonin. Adolescent rats had higher baseline extracellular serotonin than adults at the one hour time point as shown by ANOVA and post-hoc testing [Age X Time interaction serotonin $F(3,45)=3.38$, $p<0.05$, percent increase $F(3,45)=3.49$, $p<0.05$]. Fenfluramine injection produced increases in extracellular serotonin that peaked one hour after injection. Serotonin levels did not completely return to baseline during the three hours after each dose, resulting in a cumulative increase in serotonin across time [main effect of Time $F(3,45)=93.76$, $p<0.001$]. Global ANOVA indicated differential effects of the fenfluramine dose response curve in each age group with an Age X Time X Dose interaction for serotonin levels in each sample (Fig. 4a) [$F(9,135)=2.64$, $p<0.01$] and the percent increase from baseline [Fig. 4b, $F(9,135)=2.14$, $p<0.05$]. This interaction was further investigated by two factor (Age, Time) repeated measures ANOVA and post-hoc testing for each dose of fenfluramine. Significant Age X Time interactions were observed at each dose for serotonin levels [1 mg/kg $F(5,74)=2.73$, $p<0.05$, 2.5 mg/kg $F(5,75)=4.42$, $p<0.01$, 10 mg/kg $F(5,75)=3.79$, $p<0.01$] and the percent increase from baseline [1 mg/kg $F(5,74)=2.98$, $p<0.05$, 2.5 mg/kg $F(5,75)=3.96$, $p<0.01$, 10 mg/kg $F(5,75)=3.19$, $p<0.05$], indicating differential effects of each dose between age groups. Post-hoc testing showed that adolescents had lower fenfluramine-stimulated serotonin release than adults during the two peak samples following each dose.

3.4 Potassium Evoked Serotonin Release

A 20 minute infusion of 100 mM KCl into the mPFC increased serotonin levels above baseline for 40 minutes and produced a greater increase in extracellular serotonin in adult rats ($n=12$) compared to adolescents ($n=10$). ANOVA revealed an Age X Time interaction for serotonin levels (Fig. 4c) [$F(15,285)=2.37$, $p<0.01$] and percent increase from baseline (Fig. 4d) [$F(15,285)=3.64$, $p<0.001$], suggesting age differences in the response to potassium infusion. Post hoc testing showed that adolescent extracellular serotonin levels were significantly lower than those of adults at both time points of elevated serotonin following potassium infusion.

3.5 Tissue Serotonin Content and Synthesis

ANOVA for all tissue measures revealed a main effect of Treatment with NSD-1015 for 5-HIAA [$F(1,28)=38.99$, $p<0.001$], so only saline-treated animals ($n=8$ per age group) were used to investigate age differences in serotonin, 5-HIAA, 5-HIAA/5-HT. Adolescent rats had lower serotonin content (Fig. 5a–c) than adults across the prefrontal cortex, amygdala, and hippocampus [main effect of Age $F(1,14)=49.57$, $p<0.001$]. ANOVA also produced an Age X Region interaction for serotonin content [$F(2,28)=7.45$, $p<0.01$], and post hoc testing confirmed that adolescents had lower serotonin content than adults in all three brain regions. No age differences were detected in 5-HIAA levels. ANOVA confirmed brain regional differences in both serotonin [main effect of Region $F(2,28)=210.69$, $p<0.001$] and 5-HIAA [main effect of Region $F(2,28)=258.98$, $p<0.001$], with levels of each being highest in the amygdala. Adolescent rats had higher serotonin turnover (5-HIAA/5-HT) than adults [main effect of Age $F(1,14)=52.82$, $p<0.001$], which reflected the lower serotonin levels and comparable 5HIAA. Treatment with the decarboxylase inhibitor NSD-1015 increased 5-HTP levels similarly in each age group (Fig. 5d–f, $n=8$ per age, per treatment) [main effect of Treatment $F(1,28)=265.54$, $p<0.001$], indicating comparable rates of serotonin synthesis.

3.6 Serotonin Transporter Immunostaining

The density of SERT-immunoreactive innervation was quantitated in prefrontal cortex, amygdala, and hippocampus to investigate possible age differences (n=12 per age group) (Supplementary Fig. 2). There were significant regional differences in the density of SERT immunoreactive axons (Fig. 6a) [main effect Region $F(5, 104)=192.4$, $p<0.001$]. SERT immunoreactivity was highest in the basolateral amygdala, as has been seen in previous immunostaining and autoradiography studies (Hrdina et al., 1990; Sur et al., 1996). No age difference in SERT immunoreactivity was detected in these brain regions and there was no interaction of region and age.

3.7 Serotonin Transporter Radioligand Binding

We performed ^3H -paroxetine binding in homogenates of prefrontal cortex, amygdala, and hippocampus to compare total SERT levels between age groups (n=8 per age group). Single point binding with 50 pM ^3H -paroxetine (Fig. 6b) revealed effect of Age on ligand binding, though there were regional differences in binding [main effect of Region $F(2,28)=84.37$, $p<0.001$]. The amygdala had higher binding levels than cortex or hippocampus, which is consistent with the higher serotonin content and SERT immunoreactivity found in the brain region. Saturation binding of pooled homogenates revealed no age differences in B_{max} or K_d (Table 1).

4. Discussion

This study shows that adolescent male rats are less sensitive than adult males to the anxiogenic effects of the serotonin releasing drug fenfluramine, and produces neurochemical data which suggest this behavioral effect may result from lower fenfluramine-stimulated serotonin release in adolescents. Fenfluramine did not induce anxiety-like behavior as effectively in adolescent rats as in adults in either the LD test or EPM. The anxiogenic effects of fenfluramine in adult rats are consistent with previous studies showing anxiogenic effects produced by acute treatment with indirect serotonergic agonists and may represent a form of behavioral inhibition relevant to risk taking (Drapier et al., 2007; Gomes et al., 2009; Graeff et al., 1996; Griebel et al., 1994; Morley et al., 2005; reviewed in Griebel, 1995). Microdialysis in mPFC revealed lower fenfluramine-stimulated increases in extracellular serotonin levels in adolescents than in adults, suggesting that lower serotonin release in adolescents could be a mechanism underlying the insensitivity to fenfluramine's behavioral effects.

The lower fenfluramine- and potassium-stimulated serotonin release in adolescent mPFC may reflect functional immaturity of the serotonergic system. Lower serotonin content in forebrain regions has been observed in several studies of adolescent rats, and may explain the smaller increase in extracellular serotonin observed after both fenfluramine and potassium (Loizou, 1972; Loizou and Salt, 1970; Mercugliano et al., 1996). Fenfluramine stimulates non-exocytic serotonin release that is independent of serotonergic neuron firing, and may be limited by serotonin content (Carboni and Di Chiara, 1989; reviewed in Sulzer et al., 2005). The lower tissue serotonin content seen in adolescents suggests that stimuli such as fenfluramine and potassium that strongly recruit tissue serotonin stores should stimulate greater serotonin release in adults than adolescents. While lower tissue serotonin content in adolescents may result in lower fenfluramine-stimulated serotonin release, it does not create a deficit in baseline extracellular serotonin. This is consistent with reports that deficits in tissue serotonin content of at least 60% are needed to see changes in basal extracellular serotonin, as adolescent rats had only 29% lower serotonin content in prefrontal cortex (Hall et al., 1999). Lower tissue serotonin stores are not due to lower synthesis in adolescents, as shown by similar 5-HTP increases in each age group after decarboxylase

inhibition. This result is consistent with prior studies showing that tryptophan hydroxylase activity matures around PN30 in rats (Deguchi and Barchas, 1972; Park et al., 1986; Schmidt and Sanders-Bush, 1971). The immature serotonin tissue content and lower serotonin release in adolescents may reflect lower vesicular serotonin stores in the terminals of serotonergic neurons.

Comparable SERT-immunoreactive innervation density and ^3H -paroxetine binding between age groups rule out serotonergic innervation density as a contributing factor to the lower response to fenfluramine in adolescents. Mature density of serotonergic innervation was found by PN21 in a previous study of serotonin-immunoreactive neurons, although some studies have found lower SERT binding in the adolescent cortex by autoradiography (Dao et al., 2011; Lidov and Molliver, 1982; Loizou, 1972; Moll et al., 2000). The discrepancy between SERT binding in cortical homogenates in the present study and cortical autoradiography data from other studies may be explained by differences in the radioligand used or the greater anatomic resolution of autoradiography versus homogenate binding. Studies on SERT binding in subcortical structures in adolescence have also produced mixed results, so adolescent deficits in SERT expression may be relatively subtle (Dao et al., 2011; Galineau et al., 2004; Moll et al., 2000; Tarazi et al., 1998). Data on adolescent tissue serotonin content, innervation density, and SERT binding collectively indicate that early adolescent rats have a similar density of forebrain serotonergic innervation as adults, but that these terminals have lower serotonin stores.

The anxiogenic effects of fenfluramine are thought to be caused by increases in extracellular serotonin (Graeff et al., 1996). Serotonergic mediation of fenfluramine's anxiogenic effects is consistent with observations that acute treatment with indirect serotonin agonists generally produces anxiogenic effects in unconditioned tests for anxiety-like behavior (Drapier et al., 2007; Gomes et al., 2009; Graeff et al., 1996; Griebel et al., 1994; Morley et al., 2005; reviewed in Griebel, 1995). Fenfluramine also releases dopamine and norepinephrine, though at the dose used for behavior testing there is very little stimulated dopamine release and release of norepinephrine is much lower than serotonin (Balcioglu and Wurtman, 1998; Rothman et al., 2003). However, some contribution of norepinephrine to age differences in fenfluramine's anxiogenic effect is possible, as the noradrenergic system is immature through mid-adolescence in rats (reviewed in Bylund and Reed, 2007).

There may also be a postsynaptic component to the lesser anxiogenic effects of fenfluramine in adolescents. Most evidence indicates that serotonin receptor expression is mature during adolescence, and that adolescents and adults exhibit similar sensitivity to serotonin syndrome induced by serotonin receptor agonists (Beique et al., 2004; Darmani and Ahmad, 1999; Daval et al., 1987; Garcia-Alcocer et al., 2006; Li et al., 2004; Miquel et al., 1994; Pranzatelli and Galvan, 1994; Vizuete et al., 1997; Waeber et al., 1996; Waeber et al., 1994). However, connections between brain regions that mediate anxiety-like behavior such as prefrontal cortex and amygdala continue to mature during adolescence in both rodents and humans (reviewed in Casey et al., 2008; Cressman et al., 2010; Cunningham et al., 2002; Ernst et al., 2006). Adolescent behavioral insensitivity to fenfluramine could be caused by immaturity of the neural circuits modulated by serotonin, as well as lower fenfluramine-stimulated extracellular serotonin levels.

These findings have implications for neurochemical and behavioral responses to drugs of abuse during adolescence, particularly psychostimulants. Adolescent rats are less sensitive than adults to the aversive effects of psychostimulants, and may find them more rewarding based on conditioned place preference studies (Infurna and Spear, 1979; Schramm-Sapyta et al., 2006; reviewed in Schramm-Sapyta et al., 2009). Psychostimulants increase extracellular levels of dopamine, serotonin, and norepinephrine in the brain, with the relative increase in

each neurotransmitter differing between stimulants. Dopamine is primarily responsible for the reinforcing effects of psychostimulants, while norepinephrine and serotonin mostly mediate the aversive effects of these drugs (Chen et al., 2006; Jones et al., 2009; Jones et al., 2010; Lyness et al., 1979; Roberts et al., 1977; Thomsen et al., 2009). Serotonin contributes to the aversive effects of psychostimulants in animal models such as conditioned taste aversion, conditioned place aversion, and the runway model of cocaine self administration (Ettenberg and Bernardi, 2006, 2007; Ettenberg et al., 2011; Jones et al., 2009; Jones et al., 2010; Rocha et al., 2002; Serafine and Riley, 2010). Serotonin depletion studies show that serotonin inhibits psychostimulant self-administration and locomotion in rodents (Hollister et al., 1976; Loh and Roberts, 1990; Lyness et al., 1980; Mabry and Campbell, 1973). The lower fenfluramine and potassium stimulated serotonin release observed in adolescents suggest that adolescents would also have a lower serotonergic response to psychostimulants such as amphetamine, methamphetamine, and MDMA that release serotonin by the same mechanism as fenfluramine (reviewed in Sulzer et al., 2005). Methamphetamine produces similar increases in extracellular serotonin in prefrontal cortex of PN45-50 rats and adults, but this response may be immature in younger adolescent rats around PN28 (Staiti et al., 2011). This potentially lower serotonin response in adolescents could contribute to the reduced aversive effects of serotonin-releasing psychostimulants observed in this age group (Infurna and Spear, 1979). Less serotonergically-mediated aversive psychostimulant effects in adolescents could facilitate drug abuse by shifting the balance of rewarding and aversive effects toward rewarding effects in adolescents. This could be especially problematic given evidence that adolescents are more sensitive to the rewarding effects of psychostimulants in conditioned place preference models (reviewed in Schramm-Sapota et al., 2009).

The mechanism by which psychostimulants increase extracellular monoamines could be an important factor in the balance of rewarding and aversive effects in adolescents. Psychostimulants include drugs such as cocaine that increase extracellular monoamines by inhibiting uptake via transporters, as well as drugs such as amphetamine, methamphetamine, and MDMA that inhibit uptake and release monoamines by a similar mechanism as fenfluramine (reviewed in McMillen, 1983; Sulzer et al., 2005). Most studies with cocaine, an uptake inhibitor, show that adolescents are more sensitive to its conditioned rewarding effects (Aberg et al., 2007; Badanich et al., 2006; Balda et al., 2006; Brenhouse and Andersen, 2008; Brenhouse et al., 2008; Campbell et al., 2000; Schramm-Sapota et al., 2004; Zakharova et al., 2009a; Zakharova et al., 2009b). However, data from releasing drugs such as amphetamine or methamphetamine are more equivocal. Studies with these drugs have found greater rewarding effects in either age group or similar effects between groups, though adolescents self-administer more amphetamine than adults while acquiring self-administration (Adriani and Laviola, 2003; Mathews and McCormick, 2007; Shahbazi et al., 2008; Torres et al., 2008; Zakharova et al., 2009a). Even if there are no age differences in rewarding effects of releasing drugs, the reduced aversive effects of these drugs in adolescents could result in a greater rewarding to aversive effect ratio than in adults (Infurna and Spear, 1979). Comparison of the effects of uptake inhibitors versus releasers on extracellular dopamine has revealed that adolescents are more sensitive to the effects of uptake inhibitors such as cocaine upon extracellular dopamine, but not to releasing drugs such as amphetamine (Walker and Kuhn, 2008; Walker et al., 2010). This could be due to similar mechanisms as with serotonin and fenfluramine, as early adolescents have lower tissue dopamine content than adults (Porcher and Heller, 1972). Given that the rewarding/aversive effects of uptake-inhibiting psychostimulants are even more skewed toward rewarding than releasing drugs, it will be important for future studies to assess the effects of uptake inhibition on extracellular serotonin in adult and adolescent rats.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Fenfluramine was more anxiogenic to adult male rats than adolescents.
- Adolescents had lower fenfluramine-stimulated cortical serotonin release.
- Lower tissue serotonin stores may limit the effects of fenfluramine in adolescents.
- Serotonin-releasing drugs of abuse could also be less anxiogenic to adolescents.

Figure 1.

A description of the timing from arrival in our animals facility to A.) behavior testing in the LD or EPM tests, B.) microdialysis testing. Animal ages are shown in postnatal days. Adolescent rats arrived in our facilities as juveniles at PN21, while adult rats arrived as young adults aged PN60-63. All animals waited a minimum of 7 days before behavior or microdialysis testing. All experiments were timed to occur during the first week of adolescence (PN28-34).

Figure 2. Summary of behavior in the LD test. A.) Time spent in the light compartment, B.) Latency to emerge into the light compartment, C.) Number of entries into the light compartment, D.) Total distance traveled. *=significantly different from age matched controls, **=significantly different from age matched controls and fenfluramine-treated adolescents, $p < 0.05$ by Newman-Keuls post-hoc testing.

Figure 3. Summary of behavior in the EPM. A.) Percentage of total time spent in the open arms, B.) Number of open arm entries, C.) Latency to enter an open arm, D.) Percentage of total time spent in the closed arms, E.) Number of closed arm entries. *= significantly different from age matched controls, $p < 0.05$ by Newman-Keuls post-hoc testing.

Figure 4. Microdialysis results from medial prefrontal cortex. Fenfluramine-stimulated serotonin levels (A.) and percent increase from baseline (B.) and potassium-stimulated serotonin efflux (C.) and percent increase from baseline (D.). * = significant age difference at that time point, $p < 0.05$ by Newman-Keuls post-hoc testing.

Figure 5.

A–C Baseline tissue serotonin content, 5-HIAA levels, and 5-HIAA/5-HT for prefrontal cortex (A.), amygdala (B.), and hippocampus (C.). * = significant age difference, difference at that time point, $p < 0.05$ by Newman-Keuls post-hoc testing. D–F Buildup of 5-HTP after treatment with NSD-1015 (100 mg/kg) in prefrontal cortex (D.), amygdala (E.), and hippocampus (F.).

Figure 6. Density of SERT-immunoreactive projections (A.) and single point binding data for 50 pM ^3H -paroxetine (B.). A.) mPFC = medial prefrontal cortex, BLA = basolateral amygdala, CeA = central amygdala, DG = dentate gyrus, B.) PFC = prefrontal cortex, AMG = amygdala, HPC = hippocampus.

Table 1

Results of nonlinear fits for saturation binding in adult and adolescent tissue homogenates $B_{\max}$ is shown in fmol/mg of protein and K_d is shown in pM $\pm$ the standard error of the mean calculated by the nonlinear fit.

	Prefrontal Cortex	Amygdala	Hippocampus
Adolescents	$B_{\max} = 289 \pm 21$	$B_{\max} = 592 \pm 51$	$B_{\max} = 231 \pm 19$
	$K_d = 22.4 \pm 8.4$	$K_d = 38.2 \pm 14.5$	$K_d = 23.5 \pm 8.6$
Adults	$B_{\max} = 305 \pm 32$	$B_{\max} = 526 \pm 57$	$B_{\max} = 279 \pm 28$
	$K_d = 26.9 \pm 13.9$	$K_d = 35.4 \pm 16.6$	$K_d = 32.2 \pm 12.7$