Adolescent exposure to fluoxetine impairs serial pattern learning in the serial multiple choice (SMC) task in adult rats

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**ABSTRACT**

The effects of chronic adolescent fluoxetine (FLX, Prozac\textsuperscript{®}) exposure on adult cognition are largely unknown. We used a serial multiple choice (SMC) task to characterize the effects of adolescent FLX exposure on rat serial pattern learning in adulthood. Male rats were exposed to either 1.0, 2.0, or 4.0 mg/kg/day FLX for five consecutive days each week for five weeks during adolescence, followed by a 35-day drug-free period. As adults, the rats were trained in a task that required them to learn a highly structured sequential pattern of responses in an octagonal chamber for water reinforcement. In a transfer phase, the terminal element of the pattern was replaced by a violation element that was inconsistent with previously learned pattern structure. Results indicated that adolescent FLX exposure caused differential learning deficits for different types of elements in the serial pattern. Adolescent exposure to 1.0 or 4.0 mg/kg/day FLX, but not 2.0 mg/kg/day FLX, impaired chunk-boundary element learning, which is known to be mediated by stimulus-response (S-R) learning. All three doses of FLX impaired violation element learning, which is known to be mediated by multiple-cue learning. FLX did not impair within-chunk element learning, which is known to be mediated by rule-learning mechanisms. The results indicate that adolescent FLX exposure produced multiple cognitive impairments that were detectable in adulthood long after drug exposure ended.

1. Introduction

Fluoxetine (FLX, Prozac\textsuperscript{®}) is a selective serotonin reuptake inhibitor (SSRI) commonly prescribed to adolescents and adults for anxiety and mood disorders. FLX is currently the only FDA approved drug for treating depression in children eight and older (Olivier, Blom, Arentsen, & Homberg, 2011; USFDA, 2014). Previous research assessing the effects of adolescent SSRI exposure on adult rodent behavior has used animal models of human depression and anxiety-like behaviors (Homberg, Schubert, & Gaspar, 2010). In contrast, the current study examined cognitive sequelae of chronic adolescent exposure to SSRIs.

To assess the effects of adolescent FLX exposure on adult cognitive processes in a rodent model of sequence learning, we used the serial multiple choice (SMC) task in adult rats (Chenoweth & Fountain, 2015, 2016; Fountain et al., 2012; Muller & Fountain, 2010; 2016). The SMC task is modeled after nonverbal sequential learning and memory paradigms in humans (see, e.g., Knopman & Nissen, 1987; Reber, 1967; and, particularly, Restle & Brown, 1970). Sequential learning in the SMC task recruits multiple cognitive and neural processes concurrently that interact to allow the rat to learn and remember complex serial patterns (Chenoweth & Fountain, 2015; Fountain et al., 2012; Muller & Fountain, 2010; 2016; Sharp, Miller-Cahill, Riccio, & Fountain, 2018). In this task, rats learn to perform a highly structured sequential pattern of responses that can be characterized as “chunks” of several responses with “chunk-boundary” elements and rule-based “within-chunk” elements. The sequential pattern may also contain a “violation” element not predicted by the pattern structure. Learning to anticipate chunk-boundary elements has been shown to depend on stimulus-response (S-
R) learning and serial position learning or timing (Muller & Fountain, 2010; Muller & Fountain, 2016; Stempowski, Carman, & Fountain, 1999). Within-chunk elements, on the other hand, are encoded via rule learning (Muller & Fountain, 2010; Muller & Fountain, 2016). Violation element anticipation is believed to rely on multiple-item memory (Kundey & Fountain, 2010; Muller & Fountain, 2010; Muller & Fountain, 2016). Both chunk-boundary and violation element learning are impaired by NMDA-receptor and muscarinic cholinergic antagonists whereas within-chunk element learning is hardly affected by either (Chenoweth & Fountain, 2015, 2016; Fountain & Rowan, 2000; Fountain et al., 2012; Fountain, Rowan, & Wollan, 2013).

The current experiment assessed the effects of adolescent FLX exposure on adult rat sequential learning. Ampuero et al. (2013) reported that exposure to 0.7 mg/kg FLX for 5 days per week for 5 weeks during adulthood produced clinically relevant plasma levels and caused memory deficits in rats. To examine the effects of chronic lower-dose adolescent FLX exposure on adult learning in the SMC task, we examined the effects of relatively low doses. Rats were exposed to 0, 1.0, 2.0, or 4.0 mg/kg FLX by i.p. injection five days per week for five weeks during adolescence. After reaching adulthood, rats were trained in the SMC task beginning on postnatal day 95 (P95). In the training phase, rats learned a pattern consisting of eight chunks with perfect pattern structure. In the transfer phase, the final element of the perfect pattern was replaced with a violation element that was inconsistent with pattern structure. As we have in past behavioral toxicology studies, we used this complex behavioral task as a screen for potential neurotoxic effects of adolescent exposure to FLX that might affect one or more of multiple cognitive systems (cf. Pickens, Rowan, Bevins, & Fountain, 2013; Renaud & Fountain, 2016; Renaud, Pickens, & Fountain, 2015; Rowan et al., 2015).

2. Methods

2.1. Animal care and drug treatment

Male Long Evans rats (n = 24) were group housed until P21 and individually housed thereafter with free access to food and 5 min access to supplemental water daily. Rats were randomly assigned to one of three treatment groups or a saline control group (n = 6 per group). Beginning on P25, rats received intraperitoneal injections of either 0, 1.0, 2.0, or 4.0 mg/kg FLX (Sigma Chemical Co.) in saline at an injection volume of 1.0 ml/kg body weight. Injections were administered five consecutive days each week for five weeks and thus mimicked the common and sometimes recommended practice of “drug holidays” for relief from unwanted side effects of SSRIs (“Should you take a drug holiday?,” 2016). Next, rats received a 35-day drug-free period. As recommended by our institutional CITI Program’s “Research in Ethics and Compliance Training,” the experiment was conducted simultaneously with another study (Rowan et al., 2015); the same group of rats served as saline-injected controls for both experiments to reduce animal use. All rats in all conditions for both experiments were run concurrently under identical conditions. We recognize that despite the animal welfare advantages of this approach, future studies should be sensitive as always to the need to replicate effects judiciously.

2.2. Apparatus

Plexiglas shaping chambers (30 × 30 × 30 cm) had stainless steel mesh floors and a nosepoke receptacle (2.5-cm diameter black PVC pipe end caps) 5 cm above the floor equipped with infrared emitter-detector pairs mounted on the sides and a cue light mounted in the rear of the receptacle. Plexiglas test chambers were octagonal in shape (15 cm wide × 30 cm tall with 40 cm separating opposing walls) with a stainless steel mesh floor. One nosepe recep tacle was centered 5 cm above the floor on each chamber wall. A 20-ml syringe water reservoir was attached by tubing to a solenoid (General Valve Corp., 20 psig, 24 V) that controlled flow to an opening at the bottom of each receptacle for water delivery.

2.3. Procedure

2.3.1. Shaping procedure

A timeline of experimental procedures is depicted in Fig. 1. Before initial training began, rats were water deprived for 36–48 h then shaped for three days. In 1-h sessions, the receptacle light was illuminated at the beginning of each trial. When the rat responded, the light was extinguished, and a water droplet was delivered. A 1-s inter-trial interval (ITI) separated shaping trials. Rats were required to complete 120 nosepokes in one daily session in order to continue to the training phase.

2.3.2. Training phase: Acquisition of a perfect pattern

Starting on P95, rats were trained on the following “perfect” serial pattern: 123-245-567-812. Digits refer to the clockwise position of the eight nosepe recep tacles. Dashes indicate 3-s ITIs that served as phrasing cues. A 1-s ITI occurred between elements within chunks. The first element of each chunk is called a “chunk-boundary element” and the two following elements are called “within-chunk elements.”

At the beginning of each trial, all receptacle lights were illuminated. If the rat’s first nosepe was correct, all receptacle lights were extinguished and a water droplet was administered in the correct receptacle as reinforcement. However, if the rat’s first response on a trial was incorrect, a correction procedure began, where all lights except for that of the correct receptacle were extinguished and water reward was administered only after the rat chose the correct receptacle. Rats completed five patterns per day for 49 days.

2.3.3. Transfer phase: Acquisition of an added violation element

In the transfer phase, a violation “8″ element replaced the final element of the pattern (underlined): 123-234-345-456-768-781-812. Rats completed five transfer patterns per day for 28 days.

2.3.4. Statistical analysis

Analysis of variance (ANOVA) was used to examine the effects of FLX on rats’ acquisition for each element type (within-chunk, chunk-boundary, and violation elements) across daily blocks of the experiment. Main effects and interactions were considered significant if p < .05. To assess differences in acquisition of pattern elements, repeated measures ANOVA was conducted on rats’ daily total correct
3. Results

3.1. Training phase: Acquisition of a perfect pattern

Analyses determined the effects of adolescent exposure to three doses of FLX compared to controls on acquisition of a serial pattern. Training phase results showed that doses of 1.0 mg/kg and 4.0 mg/kg of FLX impaired some aspects of acquisition while sparing others whereas doses of 2.0 mg/kg had no significant effect. For within-chunk acquisition (Fig. 2a), adolescent exposure to FLX had no significant effect.

For chunk-boundary element acquisition (Fig. 2b), a 4 × 49 (drug exposure × days) repeated measures ANOVA found a significant main effect of days, $F(48,960) = 47.90, p < .001$, $\eta^2 = 0.67$ and drug $F(1,20) = 3.34, p = .049$, $\eta^2 = 0.33$. Planned comparisons indicated adolescent exposure to 1.0 and 4.0 mg/kg/day FLX produced slower acquisition of chunk-boundary elements relative to saline or 2.0 mg/kg/day FLX. Although the drug × days interaction was not significant ($p > .05$), a priori comparisons examined group differences that emerged over the course of the experiment. Planned comparisons revealed that rats exposed to 1.0 mg/kg of FLX made fewer correct responses than saline controls on days 9, 13, 16, 19, 21, 24, 30–41, 43–45, and 47–49 ($p < .05$). Rats exposed to 4.0 mg/kg of FLX made fewer correct responses than saline controls on days 9, 16, 17, 19–21, 23–27, 30–33, 35, 36, 38, 39, 41, 43, and 45–49 ($p < .05$). Rats exposed to 1.0 mg/kg of FLX made fewer correct responses than rats exposed to 2.0 mg/kg of FLX on days 19, 21, 22, 30, 32–45, 47, and 49 ($p < .05$). Rats exposed to 4.0 mg/kg of FLX made fewer correct responses than rats exposed to 2.0 mg/kg of FLX on days 16, 17, 19–21, 26, 27, 30–33, 35, 36, 38, 39, 41–43, and 45–49 ($p < .05$). Lastly, rats exposed to 4.0 mg/kg of FLX made fewer correct responses than rats exposed to 1.0 mg/kg of FLX on day 37 ($p < .05$).

Multiple comparisons revealed that saline controls (mean = 17.83) made more correct responses overall than rats who were exposed to 1.0 mg/kg (mean = 23.23, $p = .03$) and 4.0 mg/kg FLX (mean = 23.40, $p = .04$), but not rats exposed to 2.0 mg/kg FLX (mean = 18.28, $p = .85$). Rats that received 2.0 mg/kg FLX made more correct responses than rats that received 1.0 mg/kg ($p = .05$) and rats that received 4.0 mg/kg FLX ($p = .04$). There was no difference in overall correct responses made between rats exposed to 1.0 mg/kg and 4.0 mg/kg FLX ($p = .94$). These results indicate doses of 1.0 mg/kg and 4.0 mg/kg of FLX significantly impaired chunk-boundary learning. There were no significant differences in the types of errors rats made on chunk-boundary elements regardless of treatment condition, therefore slower learning did not indicate a shift in learning strategy.

3.2. Transfer phase: Introduction of a violation element

Analyses determined the effects of FLX in the transfer phase on performance of within-chunk elements, on continued acquisition of chunk-boundary elements, and on acquisition of the newly added violation element. Results showed that adolescent FLX did not impair within-chunk element performance at any dose. Doses of 1.0 and 4.0 mg/kg FLX, but not 2.0 mg/kg, impaired continued acquisition of chunk-boundary elements during transfer, and all doses of FLX impaired acquisition of the violation element during transfer.

For within-chunk elements in the transfer phase (Fig. 3a), a 4 × 28 (exposure × days) repeated measures ANOVA found a significant main effect of days, $F(27,540) = 4.87, p < .001$, $\eta^2 = 0.18$. No other main effect or interactions were significant ($p > .05$). Within-chunk element acquisition was complete by the end of the training phase (Fig. 2a), and subsequently FLX had no effect on transfer phase performance of within-chunk elements (Fig. 3a).

Because chunk-boundary element acquisition was not complete by the end of the training phase, chunk-boundary acquisition continued in the transfer phase. To assess FLX effects on chunk-boundary-element acquisition in transfer, (Fig. 3b), a 4 × 28 (drug exposure × days) repeated measures ANOVA found a significant main effect of days, $F(27,540) = 14.51, p < .001$, $\eta^2 = 0.39$. Although neither the drug main effect nor the drug × days interaction was significant ($p > .05$), a priori comparisons examined whether there was evidence of day-by-day group differences not detected by the ANOVA. Planned comparisons indicated rats exposed to 1.0 mg/kg FLX made fewer correct responses than saline controls on days 2–10, 12, 13, 15–19, and 23–25 of transfer ($p < .05$). Rats exposed to 4.0 mg/kg FLX made fewer correct responses...
responses than saline controls on days 3, 5, 8–10, 12, 13, 15, 16, 18, 19, 24, and 25 (ps < .05). Rats exposed to 1.0 mg/kg FLX made fewer correct responses than rats exposed to 2.0 mg/kg FLX on days 2, 4, 7, 12, 15, 16, and 24 (ps < .05). Rats exposed to 4.0 mg/kg FLX made fewer correct responses than rats exposed to 2.0 mg/kg of FLX on days 3, 5, 10, 12, 13, 16, and 18 (ps < .05). Lastly, rats exposed to 2.0 mg/kg FLX made fewer correct responses than saline controls on days 8 and 25 (ps < .05). The results indicate that adolescent exposure to 1.0 and 4.0 mg/kg/day of FLX significantly impaired adult chunk-boundary element performance. In contrast, adolescent exposure to 2.0 mg/kg/day of FLX resulted in chunk-boundary element performance that did not differ from controls. As in the training phase, 2.0 mg/kg/day FLX during adolescence did not affect chunk-boundary element performance in the transfer phase. Analyses conducted on the types of errors made on chunk-boundary elements found no difference in types of errors made regardless of treatment condition.

For acquisition of the violation element (Fig. 3c), a $4 \times 28$ (drug exposure $\times$ days) repeated measures ANOVA found a significant main effect of days, $F(27,540) = 9.57$, $p < .001$, $\eta^2 = 0.28$, and a significant interaction for drug $\times$ days $F(81, 540) = 1.36$, $p = .028$, $\eta^2 = 0.12$. No other main effect or interactions were significant (ps $> .05$). Planned comparisons examined differences in acquisition between dose groups. Rats exposed to 1.0 mg/kg FLX made fewer correct responses than saline controls on days 2, 12, 16–19, 23, 24, and 26 (ps < .05). Rats exposed to 2.0 mg/kg FLX made fewer correct responses than saline controls on days 12, 17–19, 22, and 25 (ps < .05). Rats exposed to 4.0 mg/kg FLX made fewer correct responses than saline controls on days 12, 13, 15–18, 20, and 22–25 (ps < .05). Rats exposed to 2.0 mg/kg FLX made fewer correct responses than rats exposed to 1.0 mg/kg FLX on days 8 and 25 (ps < .05). Rats exposed to 4.0 mg/kg FLX made fewer correct responses than rats exposed to 1.0 mg/kg FLX on day 25 (p < .05). Lastly, rats exposed to 4.0 mg/kg FLX made fewer correct responses than rats exposed to 2.0 mg/kg FLX on day 27 (p < .05). Altogether, these results indicate all doses of FLX significantly impaired learning to anticipate a newly added violation element relative to controls.

4. Discussion

The results of the current experiment indicate that adolescent exposure to FLX can produce specific learning impairments in adult rats. Adolescent FLX had no effect on adult rule learning, demonstrated by normal within-chunk acquisition and performance in both phases of the experiment. In contrast, adolescent FLX caused significant deficits in chunk-boundary and violation element learning in adulthood. Adolescent exposure to 1.0 and 4.0 mg/kg FLX impaired chunk-boundary element acquisition mediated by stimulus-response learning, demonstrated by slowed acquisition of chunk boundary learning in both phases of the experiment. Paradoxically, adolescent exposure to 2.0 mg/kg FLX produced no learning impairments in chunk-boundary acquisition in either training or transfer phases—an effect we are unable to explain and one we have not observed in other adolescent drug exposure studies in this paradigm (Pickens et al., 2013; Renaud & Fountain, 2016; Rowan et al., 2015). Adolescent FLX also had significant effects on violation element acquisition in the transfer phase. During the transfer phase, when the violation element replaced the final element of the training pattern, all three doses of adolescent FLX exposure produced deficits in violation element acquisition that persisted after chunk-boundary learning reached asymptote, suggesting that FLX caused deficits in violation element learning independent from interference by chunk-boundary learning. FLX-dependent impairment of violation element acquisition implicates impairment of multiple-item memory processes (Fountain & Rowan, 1995; Muller & Fountain, 2010; Muller & Fountain, 2016).

Interpreting these results requires a brief review of the nature of serial pattern learning in the SMC task. The SMC task recruits multiple
cognitive processes concurrently in rats (Fountain et al., 2012; Muller & Fountain, 2010; Muller & Fountain, 2016) that have been shown to depend on dissociable neural systems, including NMDA and cholinergic neurotransmitter systems. In rats, these different forms of learning are differentially sensitive to acute muscarinic cholinergic or glutamatergic (NMDA) receptor blockade (Chenoweth & Fountain, 2015, 2016; Fountain & Rowan, 2000; Fountain, Rowan, & Wollan, 2013) and are differentially sensitive to deficits in adult learning caused by chronic adolescent nicotine or methylphenidate exposures (Pickens et al., 2013; Renaud & Fountain, 2016; Rowan et al., 2015). The current study found a similar dissociation of effects of adolescent FLX exposure on adult learning. Adolescent FLX also caused a significant deficit in adult acquisition of chunk-boundary elements spanning the training and transfer phases, but the most profound effect of adolescent FLX was observed as a deficit in acquisition of a newly added violation element in the transfer phase. The results of the current study collectively support the idea that adolescent FLX exposure differentially impaired specific learning systems, namely, those responsible for 1) S-R learning for chunk-boundaries and 2) multiple-cue learning for violation elements. Previous research in our lab indicates that learning to anticipate violation elements depends at least in part on intact N-methyl-D-aspartate receptor and muscarinic cholinergic receptor systems (Chenoweth & Fountain, 2015, 2016; Fountain & Rowan, 2000; Fountain et al., 2013), although the relevant neural systems have not yet been identified. Concurrently, FLX did not affect rule learning for within-chunk elements, a common finding we have interpreted as indicating learning by a distinct neural system (Chenoweth & Fountain, 2015; Fountain, 2006; Fountain et al., 2012; Pickens et al., 2013).

Although our research has shown that adolescent exposure to drugs causes distinct and dissociable deficits in adult learning in rats, localization of these cognitive mechanisms and associated deficits requires further study. Research in this line promises to elucidate (1) the mechanisms of neurotoxic action that lead to a range of cognitive deficits caused by adolescent drug exposure, including FLX, and (2) a better functional understanding of the neural underlayment of complex behavioral systems.

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