Postnatal Fluoxetine-Evoked Anxiety Is Prevented by Concomitant 5-HT$_{2A/C}$ Receptor Blockade and Mimicked by Postnatal 5-HT$_{2A/C}$ Receptor Stimulation

Ambalika Sarkar, Parul Chachra, and Vidita A. Vaidya

Background: Postnatal treatment with the selective serotonin reuptake inhibitor fluoxetine, evokes anxiety and depressive behavior in rodent models in adulthood. We examined the role of serotonin 2A (5-HT$_{2A}$), serotonin 2C (5-HT$_{2C}$) and serotonin 1A (5-HT$_{1A}$) receptors, implicated in the development of anxiety, in the behavioral consequences of postnatal fluoxetine (PNFlx).

Methods: Control and PNFlx rat pups received concomitant treatment with the 5-HT$_{2A/C}$ receptor antagonist, ketanserin, the 5-HT$_{2A}$ receptor antagonist, MDL100907, the 5-HT$_{2C}$ receptor antagonist, SB242084, or the 5-HT$_{1A}$ receptor antagonist, WAY-100635, and were tested for behavior in adulthood. The effect of postnatal treatment with the 5-HT$_{2A/C}$ receptor agonist, DOI, on anxiety behavior was examined in adulthood.

Results: Postnatal 5-HT$_{2A/C}$ receptor blockade prevented PNFlx-evoked anxiety, attenuated depressive behavior, and normalized specific gene expression changes in the prefrontal cortex. Postnatal, selective 5-HT$_{1A}$ receptor antagonist treatment blocked PNFlx-evoked anxiety and depressive behavior, whereas 5-HT$_{2C}$ receptor antagonist treatment prevented anxiety but not depressive behavior. Postnatal 5-HT$_{2A/C}$ receptor stimulation was sufficient to evoke anxiety in adulthood. Serotonin 1A receptor blockade did not alter PNFlx-evoked anxiety but resulted in anxiety in control animals, an effect attenuated by concomitant 5-HT$_{2A/C}$ receptor blockade.

Conclusions: Postnatal fluoxetine-evoked anxiety and depressive behavior, as well as specific gene expression changes in the prefrontal cortex, were prevented by 5-HT$_{2A/C}$ receptor blockade. Adult anxiety was evoked by either 5-HT$_{2A/C}$ receptor stimulation or 5-HT$_{1A}$ receptor blockade of naive control pups. Our findings implicate serotonin 2 receptors in the development of perturbed emotionality following PNFlx and suggest that an altered balance of signaling through 5-HT$_{1A}$ and 5-HT$_{2A/C}$ receptors in early life influences anxiety behavior.

Key Words: Antidepressant, DOI, 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{2C}$, ketanserin, Prozac, SSRI, WAY-100635

Serotonin (5-HT) neurotransmission influences the development of anxiety (1). Perturbations of postnatal 5-HT levels in rodents evoke lasting consequences on emotionality (2). The selective serotonin reuptake inhibitor (SSRI), fluoxetine, is among the first-line treatments for gestational, postpartum, childhood, and adolescent depression, given its favorable side-effect profile (3–6). However, clinical evidence links perinatal SSRI exposure to neonatal serotonin withdrawal symptoms and disrupted neurobehavioral development (7–9), and childhood and adolescent fluoxetine treatment is associated with enhanced anxiety, depression, and suicidal ideation (10,11). Postnatal fluoxetine (PNFlx) treatment in mouse models, considered equivalent to the third trimester of human gestation (12), results in enhanced anxiety and depressive behavior that persist across the life span (11,13–15). This is paradoxical to the anxiolytic and antidepressant responses observed in humans (16) and in rodents (17), following fluoxetine administration in adulthood. Perturbations of 5-HT levels evoke differing consequences on mood behavior based on the temporal window (11).

The temporal window of postnatal life is critical for defining the trajectory of emotional development (18–20). It is important to unveil the role of specific 5-HT receptors in mediating the behavioral effects of PNFlx. Serotonergic neurotransmission is mediated via a large family of 14 receptor subtypes that exhibit distinct developmental expression patterns and functional responses (21–23). Genetic perturbations and pharmacologic studies in rodent models indicate an important role for 5-HT$_{1A}$, 5-HT$_{2A}$, and 5-HT$_{2C}$ receptors in the development of anxiety (2,24–34). Cortical 5-HT$_{2A}$ receptor knockouts (27) and loss of 5-HT$_{2C}$ receptor function (28) exhibit anxiolytic effects. Serotonin 1A receptors, besides their postsynaptic expression, also serve as presynaptic autoreceptors that regulate 5-HT neuron firing and release (35,36). Conditional, forebrain-specific 5-HT$_{1A}$ heteroreceptor, or raphe 5-HT$_{1A}$ autoreceptor, loss of function during postnatal life, as well as postnatal pharmacologic 5-HT$_{1A}$ receptor blockade, evoke enhanced anxiety in adulthood (25,29–33). We hypothesized that PNFlx-evoked anxiogenic responses involve a key role for 5-HT$_{1A}$ and 5-HT$_{2A}$/5-HT$_{2C}$ (5-HT$_{2A/C}$) receptors, strongly implicated in the development of anxiety. Using studies in rat models, we show that 5-HT$_{2A/C}$ receptor blockade prevents PNFlx-evoked anxiety and attenuates depressive behavior. Our results indicate that postnatal 5-HT$_{2A/C}$ receptor stimulation evokes adult anxiety, and the anxiogenic effects of postnatal 5-HT$_{1A}$ receptor antagonist treatment are attenuated by concomitant 5-HT$_{2A/C}$ receptor blockade. Our findings implicate 5-HT$_{2A/C}$ receptors in the development of perturbed emotionality following PNFlx and suggest that the balance between 5-HT$_{1A}$ and 5-HT$_{2A/C}$ receptor-driven signaling within the postnatal temporal window could play an important role in the establishment of baseline anxiety states.

Methods and Materials

Animals
Male Sprague-Dawley rats bred in the Tata Institute of Fundamental Research animal facility and maintained on a 12-hour...
light-dark cycle with ad libitum access to food and water were used for all experiments. Experimental procedures followed the National Institute of Health Guide for the care and use of animals and the Committee for the Purpose of Control and Supervision of Experimental Animals and were approved by the Tata Institute of Fundamental Research Animal Ethics Committee (56/1999/CPCSEA).

**Drug Treatments**

Litters from primiparous dams were randomly assigned to control (Ctrl) or test groups, with each treatment group containing pups from two or more litters. Pups were weaned at postnatal day (P)28, and male pups were group housed into adulthood. Pups received oral administration via a feeding needle of fluoxetine (10 mg/kg; Sigma-Aldrich, St. Louis, Missouri) or vehicle (5% sucrose, Sigma-Aldrich) once daily from P2 to P21, within 3 minutes to minimize handling stress. To examine the influence of concomitant 5-HT2A/C, 5-HT2A, 5-HT2C, or 5-HT1A receptor blockade on the behavioral consequences of postnatal fluoxetine, pups received daily pretreatment with the 5-HT2A/C receptor antagonist, ketanserin tartarate (5 mg/kg, Sigma-Aldrich), the selective 5-HT2A receptor antagonist, MDL100907 (1 mg/kg; Toronto Research Chemicals, North York, Ontario, Canada), the selective 5-HT2C receptor antagonist, SB242084 (5 mg/kg, Sigma-Aldrich), the selective 5-HT1A receptor antagonist, WAY-100635 (5 mg/kg, Sigma-Aldrich), or vehicle (5% sucrose solution) orally, 30 minutes before vehicle or PNFlx treatment daily from P2 to P21. Behavioral testing on the open field test (OFT), elevated plus maze (EPM), and forced swim test (FST) was performed in adulthood. Treatment groups were 5-HT2A/C receptor blockade experiment: Ctrl, PNFlx, ketanserin (Ket), and PNFlx + Ket (OFT, EPM: n = 6–12 per group; FST: n = 5–11 per group); 5-HT2A receptor blockade experiment: Ctrl, PNFlx, MDL100907 (MDL), and PNFlx + MDL (OFT: n = 7–13 per group; FST: n = 7–9 per group); 5-HT2C receptor blockade experiment: Ctrl, PNFlx, SB242084 (SB), and PNFlx + SB (OFT: n = 6–11 per group; FST: n = 7–11 per group); 5-HT1A receptor blockade experiment: Ctrl, PNFlx, WAY-100635 (WAY), and PNFlx + WAY (OFT: n = 5–7 per group). To examine the influence of chronic postnatal treatment with the 5-HT2A/C receptor agonist, DOI (25-dimethoxy-4-iodoamphetamine), on anxiety behavior in adulthood, pups received DOI (2 mg/kg, Sigma-Aldrich) orally from P2 to P21 (PNDOI) or vehicle (5% sucrose) (Ctrl) (n = 6–8 per group). To assess the influence of concomitant 5-HT2A/C receptor blockade on anxiety behavior following postnatal 5-HT1A receptor antagonist treatment, pups received vehicle (5% sucrose solution) or ketanserin 30 minutes before administration of WAY100635 and the treatment groups were Ctrl, WAY, and WAY + Ket (n = 8–9 per group). Drug doses were selected based on prior reports (13,25,26,37,38).

**Behavioral Tests: Open Field, Elevated Plus Maze, and Forced Swim Test**

Animals were subjected to behavioral tests in adulthood. Open field test analysis for anxiety behavior (39) was performed for the following experiments: postnatal ketanserin, MDL100907, SB242084, or WAY100635 with PNFlx, postnatal DOI, and postnatal ketanserin with WAY100635 treatment. For the OFT, the arena (100 cm × 100 cm × 70 cm) was placed in a dimly illuminated room, and behavioral tests were carried out within the light phase. Animals were released at one corner of the arena and exploratory behavior was recorded with an automated tracking system for 5 minutes. All behavioral tracks were analyzed using the Ethovision tracking system (Noldus, Wageningen, Netherlands). We assessed total distance covered within the arena, path length, and time spent in the center; number of visits to the center; and number of rears in the arena. Elevated plus maze analysis for anxiety behavior (40) was performed for the following experiments: PNFlx + Ket and PNDOI. The EPM consisted of a platform elevated 50 cm from the ground with two open and closed arms (45 × 10 cm). Animals were released into the center of the maze facing the open arm, and behavioral measures were assessed for 5 minutes. Analysis was performed for total distance covered within the maze, path length, and time spent in the open and closed arms and the number of rears in the maze. Forced swim test analysis for depressive behavior (41) was performed for the following experiments: postnatal ketanserin, MDL100907, or SB242084 with PNFlx. A modified version of the FST involved habituation on day 1 for 15 minutes to a plastic cylinder (50 cm tall, 21 cm in diameter) filled with water, followed by testing on day 2 for 5 minutes. Video recordings were assessed for immobility time, latency to immobility, and number of immobility events by an investigator blind to the treatment groups.

**Quantitative Polymerase Chain Reaction Analysis**

Quantitative polymerase chain reaction analysis was performed on RNA extracted from prefrontal cortex using the Trizol method (Trizol, Sigma-Aldrich) in the following experiment: Ctrl, PNFlx, Ket, PNFlx + Ket (n = 7–12 per group) in adulthood. Complementary DNA synthesis with the High Capacity Complementary DNA Reverse Transcription Kit (Applied Biosystems, Foster City, California) was performed on RNA (2 μg/sample) following RNA quality assessment by analysis of the 260/280 optical density ratio using Nanodrop (Eppendorf, Hamburg, Germany). Quantitative polymerase chain reaction was performed with primers for the genes of interest (Table S1 in Supplement 1). Quantitation was performed using the ΔΔCt method as previously described (42) and data were normalized to hypoxanthine-guanine phosphoribosyltransferase 1 (Hprt1).

**Statistical Analysis**

Experiments with two groups were analyzed using the unpaired Student t test and experiments with three or four groups were analyzed with one- or two-way analysis of variance (ANOVA), respectively, followed by the Bonferroni post hoc test. Parametric distribution of data was assessed using the Kolmogorov-Smirnov test. Significance was determined at p < .05 (Instat and Prism, Graphpad Software Inc., San Diego, California).

**Results**

**Postnatal 5-HT2A/C Receptor Antagonist Treatment Prevents the Development of PNFlx-Evoked Adult Anxiety**

We examined whether concomitant 5-HT2A/C receptor antagonist treatment altered the development of adult anxiety following PNFlx on the OFT and EPM (Figure 1A–I). Postnatal fluoxetine treatment enhanced anxiety behavior on the OFT (Figure 1B–E) as indicated by decreased percent path length, percent time spent, and number of visits to the center of the arena. Postnatal fluoxetine-evoked anxiety on the OFT was completely prevented by concomitant ketanserin treatment (Figure 1B–E). Two-way ANOVA analysis indicated a significant PNFlx × Ket interaction for percent path length traversed (F1,31 = 6.78, p = .014), percent time (F1,31 = 5.99, p = .02), and number of visits (F1,31 = 6.34, p = .02).
Figure 1. Postnatal serotonin 2A/serotonin 2C receptor antagonist treatment prevents the development of postnatal fluoxetine-evoked adult anxiety. Shown is a schematic representation of the treatment paradigm (A). Control (Ctrl) and postnatal fluoxetine-treated (PNFlx) pups received vehicle (Veh) or ketanserin (Ket) administration daily from postnatal day (P)2 to P21 and were assessed for anxiety behavior on the open field test (OFT) (P90) and elevated plus maze (EPM) (P100) and in a separate cohort for depressive behavior on the forced swim test (FST) (P90). Shown are representative tracks on the OFT from adult Ctrl, PNFlx, Ket, and PNFlx + Ket animals (B). Postnatal Ket treatment prevented the PNFlx-evoked anxiety observed in the percent path length traversed (C), percent time spent (D), and number of visits to the center (E) of the arena. Shown are representative tracks in the open (oa) and closed arms (ca) of the EPM from Ctrl, PNFlx, Ket, and PNFlx + Ket animals (F). Postnatal Ket treatment prevented the PNFlx-evoked anxiety observed in the percent path length traversed (G) and percent time spent in the open arms (H) and the outer two thirds of the open arms of the maze (I). Shown is a schematic representation for the FST (J). PNFlx animals showed a significant increase in percent immobility time (K) on the FST, whereas the Ket group showed a significant decrease in immobility time (K). Latency to first immobility (L) and number of immobility events (M) were unaltered across groups. Data are the mean ± SEM for percent path length and percent time in the OFT (n = 6–12 animals/group) and percent path length and percent time in the open arms of the EPM (n = 7–12 animals/group), number of visits in the OFT, percent immobility time, latency to first immobility, and number of immobility events in the FST (n = 5–11 animals/group). (*p < .05 as compared with Ctrl, $p < .05 as compared with PNFlx, two-way analysis of variance and Bonferroni post hoc test).
Postnatal 5-HT<sub>2A/C</sub> Receptor Blockade Prevents both PNFLx-Evoked Anxiety and Depressive Behavior, Whereas 5-HT<sub>2C</sub> Receptor Blockade Prevents PNFLx-Evoked Anxiety but not Depressive Behavior

We next examined whether postnatal ketanserin treatment influenced PNFLx-evoked depressive behavior on the FST (Figure 1A–M). We did not observe a significant two-way ANOVA PNFLx × Ket interaction for percent immobility time (Figure 1K) on the FST. We noted significant main effects of PNFLx (F<sub>1,33</sub> = 6.28, p = .018) and Ket (F<sub>1,33</sub> = 14.86, p = .0006) for percent immobility time. The latency to immobility and number of immobility events (Figure 1LM) were unaltered across groups. These results indicate that postnatal ketanserin treatment to naive control pups evokes antidepressant-like behavioral responses on percent immobility in the FST in adulthood.

Postnatal 5-HT<sub>2A</sub> Receptor Blockade Prevents both PNFLx-Evoked Anxiety and Depressive Behavior, Whereas 5-HT<sub>2C</sub> Receptor Blockade Prevents PNFLx-Evoked Anxiety but not Depressive Behavior

We next examined the effects of postnatal treatment with selective 5-HT<sub>2A</sub> or 5-HT<sub>2C</sub> receptor antagonists on PNFLx-evoked anxiety and depressive behavior in adulthood on the OFT and FST (Figure 2A,F). Postnatal fluoxetine-evoked adult anxiety on the OFT was prevented by both the 5-HT<sub>2A</sub> receptor antagonist, MDL100907 (Figure 2B,C), and the 5-HT<sub>2C</sub> receptor antagonist, SB242084 (Figure 2G,H). Two-way ANOVA analysis indicated a significant PNFLx × MDL interaction for percent path length traversed (F<sub>1,32</sub> = 5.52, p = .024) (Figure 2C), and number of visits to the center of the arena (F<sub>1,32</sub> = 2.37, p = .13) (Figure 2J in Supplement 1). Two-way ANOVA analysis indicated a significant PNFLx × SB interaction for percent path length traversed (F<sub>1,33</sub> = 8.09, p = .008) (Figure 2G) and percent time in the center (F<sub>1,33</sub> = 5.10, p = .031) (Figure 2H). Number of visits to the center of the arena was unaltered across groups in the PNFLx + SB experiment groups (Figure 2S in Supplement 1). Total distance traversed in the OFT was unaltered across all groups (Figure 2S in Supplement 1).

Postnatal fluoxetine-evoked depressive behavior on the FST was blocked by postnatal 5-HT<sub>2A</sub>, but not 5-HT<sub>2C</sub>, receptor blockade (Figure 2D,E,I). Two-way ANOVA analysis indicated a significant PNFLx × MDL interaction for percent immobility time (F<sub>1,32</sub> = 5.21, p = .030) (Figure 2D), latency to immobility (F<sub>1,33</sub> = 4.39, p = .045) (Figure 2E), and number of immobility events (F<sub>1,32</sub> = 27.19, p < .0001) (Figure 2S in Supplement 1). Two-way ANOVA analysis indicated no PNFLx × SB interaction for percent immobility time (Figure 2I), latency to immobility (Figure 2I), or number of immobility events (Figure 2S in Supplement 1).

Postnatal 5-HT<sub>2A/C</sub> Receptor Stimulation Evokes Anxiety Behavior in Adulthood

Given that postnatal 5-HT<sub>2A/C</sub> receptor blockade prevented PNFLx-evoked anxiety, we next addressed the influence of postnatal 5-HT<sub>2A/C</sub> receptor stimulation on adult anxiety. Postnatal treatment with the 5-HT<sub>2A/C</sub> receptor agonist DOI (PNDOI) increased anxiety on the OFT and EPM in adulthood (Figure 3A–K). Postnatal DOI animals showed a significant decrease in percent path length in the open arms (Figure 3H), increased percent path length in the closed arm (Figure 3I), and a trend (p = .07) toward an increase in time spent in the closed arms (Figure 3J), with no change in percent time in the open arms (Figure 3K). Total distance traversed in the OFT (Figure 3F) and EPM (Figure 3S in Supplement 1) did not differ across groups.

Postnatal 5-HT<sub>1A</sub> Receptor Blockade Does Not Alter PNFLx-Evoked Anxiety but Evokes Anxiety in Control Animals, an Effect Attenuated by Concomitant 5-HT<sub>2A/C</sub> Receptor Blockade

We next examined whether concomitant treatment with the selective 5-HT<sub>1A</sub> receptor antagonist, WAY-100635, influenced PNFLx-mediated anxiety behavior on the OFT (Figure 4A). Postnatal WAY-100635 treatment did not alter the significant PNFLx-evoked anxiety on the OFT (Figure 4B–D). Two-way ANOVA analysis revealed no significant PNFLx × WAY interaction for percent path length traversed (Figure 4C) and percent time spent (Figure 4D) in the center of the arena. Postnatal WAY-100635 treatment to naive animals induced significant anxiety with a decline in the percent path length (Figure 4C) (main effect of WAY: F<sub>1,20</sub> = 4.51, p = .016) and percent time spent (Figure 4D) (main effect of WAY: F<sub>1,20</sub> = 9.90, p = .005) in the center of the arena. Total distance traversed in the entire arena did not differ across groups (Figure 4E).

We then assessed whether the anxiety evoked by postnatal WAY-100635 administration was influenced by concomitant 5-HT<sub>2A/C</sub> receptor antagonist treatment. Postnatal ketanserin administration partially attenuated the anxiogenic effects of postnatal WAY100635 on the OFT in adulthood (Figure 4F–I). One-way ANOVA and post hoc Bonferroni analysis indicated that while the WAY group exhibited a significant decline in percent path length traversed (Figure 4G), percent time spent (Figure 4H), and number of visits (Figure 4I) to the center of the arena, the WAY + Ket group did not differ from control animals on these measures. Total distance traversed in the arena was unaltered across groups (Figure 4J).

Postnatal Ketanserin Treatment Blocks Specific Transcriptional Changes Observed in the Prefrontal Cortex Following PNFLx Treatment

We examined the influence of PNFLx on 5-HT<sub>1A</sub> (Htr1a), 5-HT<sub>2A</sub> (Htr2a), and 5-HT<sub>2C</sub> (Htr2c) receptor expression in the prefrontal cortex, a region strongly implicated in the regulation of anxiety behavior. Quantitative polymerase chain reaction analysis revealed a significant increase in prefrontal 5-HT<sub>2A</sub> messenger RNA (mRNA) levels in PNFLx animals in adulthood, with no change observed in the other 5-HT receptors examined (Figure 5A,B). The PNFLx-evoked increase in 5-HT<sub>2A</sub> receptor mRNA was prevented by postnatal ketanserin treatment. Two-way ANOVA analysis indicated a significant PNFLx × Ket interaction for 5-HT<sub>2A</sub> receptor expression.
Postnatal serotonin 2A receptor blockade prevents both postnatal fluoxetine (PNFlx)-evoked anxiety and depressive behavior, whereas serotonin 2C receptor blockade prevents PNFlx-evoked anxiety but not depressive behavior. Shown is a schematic representation of the treatment paradigm (A). Control (Ctrl) and postnatal fluoxetine treated (PNFlx) pups received vehicle (Veh) or serotonin 2A receptor antagonist MDL100907 (MDL) from postnatal day (P)2 to P21 and were assessed on the open field test (OFT) (P90) and the forced swim test (FST) (P100). Postnatal MDL treatment prevented the PNFlx-evoked anxiety observed in the percent path length traversed (B) and percent time spent (C) in the center of the OFT arena. Postnatal MDL treatment prevented the PNFlx-evoked depressive behavior on the FST observed in percent immobility time (D) and latency to immobility (E). Shown is a schematic representation of the treatment paradigm (F). Ctrl and PNFlx pups received Veh or serotonin 2C receptor antagonist SB242084 (SB) from P2 to P21 and were assessed on the OFT and FST. Postnatal SB treatment prevented the PNFlx-evoked anxiety observed in the percent path length traversed (G) and percent time spent (H) in the center of the OFT arena. Postnatal SB treatment did not alter the PNFlx-evoked depressive behavior on the FST (I, J). Data are the mean ± SEM for percent path length and percent time in the OFT (PNFlx + MDL: n = 7–13 animals/group, PNFlx + SB: n = 6–11 animals/group), percent immobility time and latency to first immobility in the FST (PNFlx + MDL: n = 7–9 animals/group, PNFlx + SB: n = 7–11 animals/group). *p < .05 as compared with Ctrl, $p < .05 as compared with PNFlx, &p < .05 as compared with SB, two-way analysis of variance and Bonferroni post hoc test.)
We next addressed whether the expression of the immediate early gene, activity-regulated cytoskeleton-associated protein, Arc, whose levels within the prefrontal cortex are reported to be decreased in animal models of depression (43), was influenced by PNFlx treatment. Postnatal fluoxetine treatment evoked a significant decline in prefrontal Arc mRNA levels in adulthood, which was prevented by postnatal ketanserin treatment. Two-way ANOVA analysis indicated a significant PNFlx × Ket interaction for Arc expression in the prefrontal cortex ($F_{1,36} = 7.14, p = .011$). These results indicate that postnatal ketanserin treatment blocks the regulation of specific PNFlx-regulated genes in the prefrontal cortex in adulthood.

Figure 3. Chronic postnatal treatment with the serotonin 2A/serotonin 2C agonist DOI evokes anxiogenic responses in adulthood. Shown is a schematic representation of the treatment paradigms (A). Pups received treatment with either vehicle (Ctrl) or DOI (postnatal DOI [PNDOI]) daily from postnatal day (P)2 to P21 and were assessed for anxiety behavior in the open field test (OFT) (P90) and elevated plus maze (EPM) (P100). Shown are representative tracks of exploratory behavior in the open field arena from adult Ctrl and PNDOI animals with the center ‘c’ indicated by the gray zone (B). Postnatal DOI animals exhibited significant decreases in the percent path length traversed (C) and percent time spent (D) in the center of the arena. Postnatal DOI animals exhibited a trend toward a decline in the number of visits to the center of the OFT arena (E). Total distance moved in the OFT arena was unaltered between groups (F). Shown are representative tracks of exploratory behavior in the open (oa) and closed (ca) arms of the EPM from adult Ctrl and PNDOI animals (G). Postnatal DOI treatment evoked anxiety behavior in the EPM test in adulthood with significant decreases in the percent path length traversed in the open arms (H) and a significant increase in the percent path length traversed in the closed arms (I) of the maze. While percent time spent in the closed (J) and open arms (K) of the maze did not exhibit significant differences across the groups, we noted a trend ($p = .07$) toward an increase for percent time spent in the closed (J) arms of the maze. Data are the mean ± SEM percent path length, percent time and number of visits to the center, and total distance moved in the OFT and percent path length and percent time in the open and closed arms of the EPM (n = 6–8 animals/group). *$p < .05$ as compared with Ctrl (Student t test).
Discussion

The major novel finding of our study indicates that PNFlx-evoked anxiety is prevented by postnatal 5-HT$_{2A/C}$ receptor blockade and that postnatal 5-HT$_{2A/C}$ receptor stimulation behaviorally mimics the anxiogenic effects of PNFlx. Our results with selective 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor antagonists indicate that both PNFlx-evoked adult anxiety and depressive behavior are normalized.
by concomitant 5-HT2A receptor blockade, whereas 5-HT2C receptor blockade prevents the development of anxiety, but not depressive behavior, in PNFlx animals. Further, we demonstrate that postnatal 5-HT2A receptor blockade does not alter the development of anxiety following PNFlx but is sufficient to evoke adult anxiety in naive control animals, an effect diminished by 5-HT2A/C receptor antagonist co-administration. Our results implicate 5-HT2A/C receptors in mediating the behavioral effects on anxiety and depressive behavior noted in response to administration of the SSRI fluoxetine in early life. These observations provide strong support to the emerging view that a balance between 5-HT1A and 5-HT2A/C receptor-mediated signaling during the critical time window of postnatal life is of import in the emergence of anxiety.

Postnatal life represents a critical period wherein serotonergic neurotransmission is hypothesized to regulate the maturation of emotional neurocircuitry, thus programming lasting effects on emotionality (1,2,44). Genetic knockout experiments with 5-HT transporter knockout (5-HTT−/−) mice (14,45–47), as well as pharmacologic perturbations with SSRIs in rodents (13,14,48,49), indicate that elevated postnatal 5-HT levels evoke persistent increases in anxiety and depressive behavior. However, the contribution of specific 5-HT receptors to the persistent anxiogenic and depressive behaviors programmed by elevated 5-HT levels during postnatal development is poorly understood. Our pharmacologic studies provide novel evidence that implicate 5-HT2A and 5-HT2C receptors in mediating specific behavioral consequences of SSRI treatment in postnatal life.

Mouse mutant studies strongly implicate 5-HT1A, 5-HT2A, and 5-HT2C receptors in the development of anxiety (2,50–52). Anxiolytic effects have been noted in both 5-HT2A and 5-HT2C receptor knockout mice (27,28). Conditional reactivation of the cortical 5-HT2A receptor prevented the anxiolytic effects in 5-HT2A receptor knockout mice; however, it is unknown whether there is a critical temporal window for such a rescue (27). In this regard, it is of note that our studies with postnatal 5-HT2A/C, 5-HT2A, or 5-HT2C antagonist treatment did not influence anxiety behavior in adulthood in naive control animals but were each capable of preventing the development of PNFlx-evoked anxiety. In contrast, PNFlx-mediated depressive behavior on the FST was prevented by selective 5-HT2A receptor but not 5-HT2C receptor blockade in postnatal life. Baseline FST behavior in naive control animals was not significantly altered following postnatal 5-HT2A or 5-HT2C receptor blockade; however, we did observe significant antidepressant-like responses in control animals postnataally administered the 5-HT2A/C receptor antagonist. These results support the notion that while both 5-HT2A and 5-HT2C receptors contribute to the emergence of anxiety, 5-HT2A receptors may mediate the emergence of depressive behaviors following early SSRI exposure. In addition to genetic knockout studies that implicate 5-HT2A/C receptors in the regulation of anxiety (2,27–30), recent reports demonstrate that models of psychiatric vulnerability based on early adverse experience, including maternal separation (53) and prenatal exposure to influenza (54), exhibit enhanced 5-HT2A/C receptor mediated signaling. Strikingly, chronic postnatal ketanserin treatment overlapping with the period of maternal separation prevents the development of anxiety and the dysregulated cortical immediate-early gene responses in maternally separated animals (26). This suggests the possibility that both adverse early experience, as well as pharmacologic treatments with SSRIs in postnatal life, may impinge on 5-HT2A/C receptor signaling to program adult anxiety.

Further support for a role of 5-HT2A/C receptors in PNFlx-evoked anxiety comes from our results demonstrating that chronic postnatal treatment with the 5-HT2A/C receptor partial agonist, DOI, also elicits adult anxiety. In contrast to the anxiogenic effects observed with chronic postnatal DOI treatment, acute, but not chronic, adult DOI administration is thought to evoke anxiolytic effects (55–57). The effects of 5-HT2A/C receptor stimulation on anxiety behavior during postnatal versus

**Figure 5.** Postnatal ketanserin (Ket) treatment blocks specific transcriptional changes observed in the prefrontal cortex following postnatal fluoxetine (PNFlx) treatment. Shown is a schematic representation of the treatment paradigm (A). Control (Ctrl) and postnatal fluoxetine-treated (PNFlx) pups received vehicle or Ket administration from postnatal day (P)2 to P21 and were subjected to gene expression analysis in the prefrontal cortex in adulthood (P110). Postnatal Ket treatment prevented the PNFlx-evoked increase in Htr2a expression (B) and the PNFlx-evoked decline in Arc expression in the prefrontal cortex (C). Data are represented as the fold change of control animals and are the mean ± SEM (n = 8–12 animals/group). (*p < .05 as compared with Ctrl, $p < .05 as compared with PNFlx, two-way analysis of variance and Bonferroni post hoc test). 5HT, serotonin; mRNA, messenger RNA.
adult life appear distinct in nature, though the caveat that the duration of treatments varies has to be kept in mind. Adult-onset treatment with 5-HT_{2A/C} receptor antagonists (58–61) or intra-cerebroventricular administration of 5-HT_{2A} antiserine oligonucleotides also evokes anxiolytic effects (62). In addition to cortical 5-HT_{2A} receptors, 5-HT_{2A/C} receptors within other brain regions, including the hypothalamus, amygdala, and periaqueductal gray, are also implicated in the regulation of anxiety (63–65). The anxiolytic effects observed with both adult 5-HT_{2A/C} receptor agonists and antagonists may involve specific contributions of these receptors in multiple brain circuits that regulate anxiety. Our results demonstrate that during the postnatal window, 5-HT_{2A/C} receptors may play an important role in programming adult anxiety. However, at present, it is difficult to delineate the specific neuronal circuits that underlie the behavioral effects of postnatal 5-HT_{2A/C} receptor agonist treatment and contribute to the 5-HT_{2A/C} receptor antagonist-mediated normalization of PFNflx-evoked anxiety and depressive behaviors. The pattern of expression of the 5-HT_{1A}, 5-HT_{3A}, and 5-HT_{2C} receptors shows a developmental regulation with high levels observed in several cortical brain regions during postnatal life (66–72). Cortical excitatory responses to 5-HT, driven via the 5-HT_{2A} receptor, are noted during postnatal life within layer V output neurons of the prefrontal cortex, which gradually tip into predominantly inhibitory responses to 5-HT, mediated by the 5-HT_{1A} receptor, as animals mature (70–73). This suggests a transient postnatal window wherein elevated levels of 5-HT, possibly through cortical 5-HT_{2A} receptor-mediated responses, may exert a strong influence on the development of emotional neurocircuitry, such as the prefrontal cortex. Enhanced prefrontal 5-HT_{2A} receptor expression in PFNflx animals in adulthood, which was prevented by postnatal 5-HT_{2A/C} receptor blockade, raises the intriguing possibility of a tipping toward enhanced 5-HT_{2A} receptor-driven responses, as has been noted in other models of psychiatric vulnerability (53,54). Postnatal fluoxetine animals also exhibited a significant decline in the expression of the activity responsive immediate early gene, Arc, in the prefrontal cortex, which was prevented by concomitant 5-HT_{2A/C} receptor blockade. Previous studies indicate a decline in prefrontal Arc expression in susceptible mice following social defeat and in depressed patients (43). Such an alteration in immediate early gene expression may be indicative of altered cortical excitability following PFNflx. Future studies are required to identify the contribution of specific neuronal circuits to the behavioral consequences of PFNflx, and to address whether adult-onset 5-HT_{2A/C} receptor blockade can alter the trajectory of PFNflx-evoked emotional behaviors.

Genetic perturbations and pharmacologic studies indicate a role for cortical 5-HT_{1A} heteroreceptors and raphe 5-HT_{1A} autoreceptors in the developmental programming of anxiety and depressive behaviors (29,30,32). While our results confirmed prior reports (25) that postnatal 5-HT_{1A} receptor antagonist treatment enhances anxiety in control groups, it did not alter PFNflx-evoked anxiety behavior. Interestingly, the postnatal WAY-100635-evoked anxiety in naive control animals was attenuated by concomitant 5-HT_{2A/C} receptor blockade, suggesting an interaction between these 5-HT receptors during postnatal life in the development of anxiety. Immunohistochemical (74) and electrophysiological studies (75,76) indicate co-localization of 5-HT_{1A} and 5-HT_{2A} receptors in prefrontal glutamatergic neurons, and prior studies suggest a functional interaction between these receptors in the regulation of threat perception and prefrontal regulation of amygdala reactivity (77).

Taken together, our results indicate that 5-HT_{2A} and 5-HT_{2C} receptors contribute to the anxiety and depressive behaviors evoked by PFNflx and suggest that an optimal balance between 5-HT_{1A} and 5-HT_{2A/C} receptor-mediated drive during the postnatal temporal window may be required for the programming of normal levels of anxiety. We posit that SSRI treatments within this temporal window may disrupt the balance between 5-HT_{1A} and 5-HT_{2A/C} receptor function and could serve to tip the system toward enhanced 5-HT_{2A/C} receptor-mediated drive, thus contributing to the establishment of persistent increases in anxiety noted long after the cessation of SSRI treatment. It is tempting to speculate that multi-action drugs that exhibit combined 5-HT_{1A} agonist and 5-HT_{2A/C} antagonist properties may serve as putative therapeutic agents for the treatment of early-onset anxiety disorders (78,79). Our results provide impetus for future studies to address the key role of 5-HT_{2A} and 5-HT_{2C} receptors and their downstream targets in contributing to the behavioral effects of early SSRI administration.

This research was supported by a Tata Institute of Fundamental Research intramural grant (VAV). The authors report no biomedical financial interests or potential conflicts of interest.

Supplementary material cited in this article is available online at http://dx.doi.org/10.1016/j.biopsych.2013.11.005.


www.sobp.org/journal