Introduction

Fluoxetine (FLX) is the most commonly prescribed selective serotonin reuptake inhibitor (SSRI) and has been in clinical use since the late 1980s (Perez-Caballero et al., 2014). This highly lipophilic drug crosses the blood-brain barrier and selectively blocks the serotonin transporter in the brain, thus inhibiting serotonin reuptake from the synaptic cleft (Perez-Caballero et al., 2014). This leads to increased serotonergic neurotransmission resulting in antidepressant effects (Perez-Caballero et al., 2014). FLX is also widely prescribed to treat depression during pregnancy, despite a lack of knowledge regarding both treatment efficacy and risks for the fetus and child (Pei et al., 2016). Although FLX passes through the placental barrier, little is known about how it affects (neuro)-developmental outcomes in children exposed in utero (Rampono et al., 2009; Pei et al., 2016).

Several studies have found an association between antenatal SSRI exposure and increased risks for autism spectrum disorders (ASD) in children. Three recent systematic reviews and meta-analyses of large case-control and cohort studies found associations between in utero SSRI exposure and an increased risk (1.5- to 2-fold) for ASD (Man et al., 2015; Andalib et al., 2017; Kaplan et al., 2017) and two others found associations between maternal use of antidepressants (including SSRIs) during pregnancy and increased risk for ASD in children (Rais and Rais, 2014; Mezzacappa et al., 2017). A number of rodent studies have shown reduced play behavior

ABBRVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA, analysis of variance; ASD, autism spectrum disorder; BERKO, ERβ knockout; DA, dopaminergic; En1, homeobox engrafted-1; En2, homeobox engrafted-2; ER, estrogen receptor; FLX, fluoxetine; 5-HT, 5-hydroxytryptamine; Lmx1a, LIM homeobox transcription factor 1 α; mDA, midbrain dopaminergic; mDPC, midbrain dopaminergic precursor cell; mESC, mouse embryonic stem cell; Nes, nestin; NPC, neural precursor cell; Nkx2.2, NK2 homeobox homebox 2; Otx2, orthodenticle homebox 2; Pax3, paired box 3; Pitx3, paired-like homeodomain transcription factor 3; Reln, reelin; Rora, retinoic acid receptor-related orphan receptor-α; SSRI, selective serotonin reuptake inhibitor; Th, tyrosine hydroxylase; Tubb3, β3-tubulin; WT, wild-type.
and social motivation following perinatal administration of SSRIs (fluoxetine and citalopram), which is suggestive of neurodevelopmental disorders with impaired social behaviors, such as in ASD (Olivier et al., 2011; Rodriguez-Porcel et al., 2011; Simpson et al., 2011; Khatri et al., 2014).

ASD is a neurodevelopmental disorder characterized by deficient social communication and interaction, as well as repetitive behavioral and activity patterns (American Psychiatric Association, 2013). The causes of ASD have not been fully elucidated, but the etiology of this complex disorder is known to involve a combination of genetic and environmental factors that underlie dysfunctions in the amygdala, prefrontal cortex, and nucleus accumbens (Chaste and Leboyer, 2012; Park et al., 2016). These brain areas are associated with social behavior and social

| Primer | Forward | Reverse |
|--------|---------|---------|
| 36B4   | GACACCTCCAGGAAGCGA | AGTGTCGCAAATGGCAGCAT |
| Erα    | TGCTCAGCAGAAGAAGAG | CACAGTACGAGGCTCCTGAG |
| Erβ    | AGGCTTAAACAGAGAAGAC | ATGTACTGGATGCAGCAT |
| Tubb3  | TGCATCCCTTATGTGTCCT | AGCTCTGAACTTGCGTAC |
| Nes    | AGGAGGAAAGCGGAGGAGAAG | CACAGTACGAGGCTCCTGAG |
| En1    | ATGTACGAGGCTTTCAGGAG | AGTGTCGCAAATGGCAGCAT |
| Otx2   | GACACCCTCCAGGAAGCGA | AGTGTCGCAAATGGCAGCAT |
| Nkx2.2 | AGTGACCTGTGCCAAATGGAC | TTGGCGCGAAGGAGCAGAAG |
| Lmx1a  | AGGAGGAAAGCGGAGGAGAAG | CACAGTACGAGGCTCCTGAG |
| Pitta3 | AGGCTCTGAGAAGAAGAC | AGTGTCGCAAATGGCAGCAT |
| Th     | ATGTACGAGGCTTTCAGGAG | AGTGTCGCAAATGGCAGCAT |
| SRET1  | ATGTACGAGGCTTTCAGGAG | AGTGTCGCAAATGGCAGCAT |
| Tph2   | ATGTACGAGGCTTTCAGGAG | AGTGTCGCAAATGGCAGCAT |
| Tbxd2  | ATGTACGAGGCTTTCAGGAG | AGTGTCGCAAATGGCAGCAT |
| Fkbp2  | ATGTACGAGGCTTTCAGGAG | AGTGTCGCAAATGGCAGCAT |
| Slc6a3 | ATGTACGAGGCTTTCAGGAG | AGTGTCGCAAATGGCAGCAT |
| Nes    | AGGCGAATCTGGGAACTTG | AGGGTTTCCCATCTGCAAAGG |
| En1    | ATGTACGAGGCTTTCAGGAG | AGTGTCGCAAATGGCAGCAT |
| Otx2   | ATGTACGAGGCTTTCAGGAG | AGTGTCGCAAATGGCAGCAT |
| Nkx2.2 | AGTGACCTGTGCCAAATGGAC | TTGGCGCGAAGGAGCAGAAG |

Foxp2, Forkhead box protein P2; Gria2, glutamate ionotropic receptor AMPA type subunit 2.

Fluoxetine Affects Dopaminergic Differentiation In Vitro

**Fig. 1.** Dopaminergic marker expression in mDPCs. (A) Th and SRET1 mRNA expression relative to Rplp0 expression in mDPCs treated with solvent (CTRL) or with 0.1 μM FLX for 6 days. (B) Representative images of Th immunoreactivity in mDPCs upon control (CTRL) or 0.1 μM FLX treatments. Right panels represent quantifications (mean gray value). Yellow box insets in (B) representing spindle-shaped (in 0.1 μM FLX) and normally branched Th positive cells (in CTRL) are enlarged in right-most column. (C) Neurite length measurements of Th positive cells in control (CTRL) or 0.1 μM FLX-treated mDPCs. Data represent means ± CI from three independent experiments in triplicates. Significant differences [two-way ANOVA for (A); t test for (B) and (C)] as compared with control are marked with asterisks (*P < 0.01; **P < 0.001). Scale bar, 100 μm in (B).
reward response and cognitive, language, and emotion processing, all of which are deficient in autistic patients (Park et al., 2016). ASD is also linked albeit partially to hereditary components (about 50%) (Hallmayer et al., 2011), suggesting that environmental factors and toxicant exposure may play a more crucial role than initially considered (Varshney and Nalvarte, 2017).

The social motivation hypothesis of autism was developed after observations of decreased motivation to attend to social stimuli in autistic children (Scott-Van Zeeland et al., 2010). This implies dysfunctions in the brain’s reward circuitry, which, in turn, has been linked to dysfunctions in dopaminergic (DA) signaling (Scott-Van Zeeland et al., 2010; Dichter et al., 2012; Pavl, 2017). In mammals, the two main clusters of neurons that secrete dopamine are located in the midbrain and project to the nucleus accumbens, the prefrontal cortex, and the caudate putamen (Dichter et al., 2012). The development of midbrain DA (mDA) neurons is orchestrated through the activity of transcription factors and morphogens that regulate midbrain patterning and specification of midbrain progenitor cells (Arenas et al., 2015).

To date there are no studies addressing the effects of prenatal FLX exposure on the mDA differentiation process as a possible source of abnormal DA neurotransmission during development. As mentioned above, mDA neurons are the main source of DA projections in the mammalian brain, and their early differentiation, in addition to maturation, could be critical for normal DA neurotransmission in the mesocorticostrial and nigrostriatal pathways.

Mouse embryonic stem cells (mESC) offer a reliable and validated in vitro model for the assessment of prenatal toxicity of chemicals, including drugs (Bremer and Hartung, 2004). Recently, a well-defined mESC neural differentiation model (Lee et al., 2000) was successfully used to generate mDA neurons from mESCs in vitro, a process that shown to be, to a certain extent, dependent on ERβ expression (Varshney et al., 2017). For the present study, mDA neural precursors generated from mESCs were used to assess the

Fig. 2. FLX affects factors essential for midbrain patterning, specification, and mDA neurogenesis. WT NPCs were differentiated into mDPCs and concomitantly treated with 1, 0.1, and 0.01 μM FLX. Subsequently, mRNA expression of Otx2 (A), Nkx2.2 (B), En1 (C), and En2 (D) was measured using qPCR. (E) Representative images of immunohistochemistry of En1 in control and FLX-treated midbrain precursors. Right panel represents quantification (mean gray value) of (E). (F) Expression of Pitx3 and (G) Lmx1a upon control FLX treatments. Data represent means ± confidence interval (CI) from three independent experiments in triplicates. Significant differences one-way ANOVA, (A)–(D, F, G), and t test, (E) compared with control are marked with asterisks (**P < 0.01; ***P < 0.001). Scale bar, 100 μm in (E).
effect of therapeutically relevant doses of FLX on the expression of key factors involved in midbrain fate specification. As this cell model lacks serotonergic synapses and considering previous evidence that FLX can affect estrogen receptor mRNA levels and transcriptional activity (Foran et al., 2004; Mennen et al., 2008, 2010; Lister et al., 2009; Schultz et al., 2011; Pop et al., 2015), we also addressed the involvement of ERs as a potential mode of action in FLX’s effects.

**Materials and Methods**

**Culture and Maintenance of Neural Progenitor Cells.** Wild-type (WT) and ERβ-knockout (BERKO) neural precursor cells (NPCs) were derived from mESCs as described earlier (Lee et al., 2000; Varshney et al., 2017). NPCs were grown in neural expansion media containing KnockOut DMEM/F-12 (without glutamine and HEPES; Life Technologies/Thermo Fisher Scientific, Paisley, UK) supplemented with 1% N-2 Max (R&D Systems, Abingdon, UK), 0.1% gentamycin (from a 50 mg/ml solution; Life Technologies), 8.6 mM glucose (Life Technologies), 0.5 mM L-glutamine (Life Technologies), 20 mM NaHCO3 (Life Technologies), 10 ng/ml FGF basic (PeproTech, Rocky Hill, NJ), and 1 μg/ml laminin (Novus Biologicals, Abingdon, UK). NPCs were plated on poly-L-ornithine (50 μg/ml; Sigma-Aldrich, Steinheim, Germany) and laminin (1 μg/ml; Novus Biologicals)-coated dishes at a seeding density of 6 × 10^4 to 1 × 10^6 cells/cm². Media was changed every day and cells were passaged at confluency doubling in each cycle. Threshold cycle values for targets were determined from standard dilutions of test samples. Primer efficiency was 2 in most of the sets used, representing a primer set using standard curves with serial dilutions of test samples. PCR efficiency was calculated for each primer set using standard curves with serial dilutions of test samples. Primer efficiency was 2 in most of the sets used, representing a primer set using standard curves with serial dilutions of test samples. Primer efficiency was 2 in most of the sets used, representing a primer set using standard curves with serial dilutions of test samples. Primer efficiency was 2 in most of the sets used, representing a primer set using standard curves with serial dilutions of test samples. Primer efficiency was 2 in most of the sets used, representing a primer set using standard curves with serial dilutions of test samples. 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Primer efficiency was 2 in most of the sets used, representing a primer set using standard curves with serial dilutions of test samples. Primer efficiency was 2 in most of the sets used, representing a primer set using standard curves with serial dilusions of test samples. Primer efficiency was 2 in most of the sets used, representing a doubling in each cycle. Threshold cycle values for targets were determined in triplicates and P0 large ribosomal protein (Rplp0) (known as 36B4) was used as a reference gene. Relative fold changes were calculated using the ΔΔCt method.

**Immunocytochemistry and Fluorescence Imaging.** Cells were grown on poly-L-ornithine and laminin-coated chambered culture slides (Ibidi, Martinsried, Germany) and fixed in ice-cold 4% paraformaldehyde (Santa Cruz Biotechnology, Dallas, TX) for 15 minutes followed by three washes with phosphate-buffered saline (PBS).
pH 7.4], incubated with blocking serum (1% bovine serum albumin and 0.1% Tritonx-100; both from Sigma-Aldrich) in PBS and stained with primary antibodies against Th (R&D Systems, Minneapolis, MN), En1 (Sigma-Aldrich), SERT (Thermo Fisher Scientific, Waltham, MA) followed by detection with Alexa Fluor secondary antibodies (Thermo Fisher Scientific) and DAPI (300 nM; Molecular Probes, Eugene, OR) with PBS washes between and after each incubation. Slides were observed under Axioplan2 epifluorescent microscope (Carl Zeiss, Oberkochen, Germany) using 20°C objective (Fluor-Plan) with appropriate filter sets for each fluorophore and images were captured with Zeiss Axiocam MR scientific camera using Axiovision 4.0 software (Carl Zeiss) Appropriate negative and positive controls were incorporated for respective primary and secondary antibodies.

**Image Analysis.** Quantification of immunoexpression density was performed by analyzing images in Fiji (ImageJ; National Institutes of Health, Bethesda, MD) image analysis software, using similar settings for each marker across all images and experimental conditions. Neurite length measurements were performed on calibrated images using NeuronJ plugin in Fiji (Meijering et al., 2004) with length threshold above 5 μm.

**In Silico ER Binding Predictions.** A molecular docking approach was used to detect potential interactions between FLX and the ERs. The X-ray structures of ERα and ERβ (Protein Data Bank ID: 1sj0 and 2fsz respectively) were acquired from the “A Database of Useful Decoys: Enhanced” [http://dude.docking.org/] database (Mysinger et al., 2012). A dataset of ER ligands containing 131 active and 101 inactive compounds was obtained from the U.S. Food and Drug Administration’s Endocrine Disruptor Knowledge Base (EDKB) (Ding et al., 2010). The protein structures were prepared for docking using the Schrödinger Protein Preparation Wizard and the compounds from the EDKB ER dataset were prepared using the default settings of Schrödinger LigPrep. Docking simulations were performed using Schrödinger Glide (Friesner et al., 2004), with the default settings of the Glide SP (Single Precision) tool. The docking scores were then subsequently binned into 0.5 bins.

![Fig. 4. FLX treatment of mDPCs affects some genes associated with autism. Relative change in mRNA expression levels of (A) Foxp2, (B) Pax3, (C) Gria2, (D) Rora, and (E) Reln for control (CTRL) or 1, 0.1, and 0.01 μM FLX treatments in mDPCs. Data represent means ± CI from three independent experiments in triplicates. Significant differences (one-way ANOVA) as compared to control are marked with asterisks (** for P < 0.01; *** for P < 0.001).](image-url)
Statistical Analysis. Experiments were performed three times with three technical replicates. If not stated otherwise, the statistical differences between groups were analyzed using the GraphPad Prism 6 software by one-way analysis of variance (ANOVA), followed by Dunnett’s test for multiple comparisons, or by Student’s t test for single comparisons. P values <0.05 were considered statistically significant unless stated otherwise. Data are shown as means ± 95% confidence intervals.

Results

FLX Affects the Differentiation of NPCs to Midbrain Precursors. To assess if FLX affects early differentiation of the midbrain, we used a well-defined mESC model that mimics neural development in vitro, which is marked by the presence of early generating neural precursor cells (NPCs) and subsequent induction of midbrain dopaminergic precursor cells (mDPCs). Although immature, these mDPC cultures contain dopaminergic neuronal precursors that express tyrosine hydroxylase (TH) as well as low levels of the dopamine transporter (SLC6A3) (Fig. 1A). However, these cells are nascent and do not form synapses yet. Using this cell model, we analyzed the expression of genes that are critical for the early development of mDPCs, as well as mDA neurogenesis, following treatment with therapeutically relevant FLX concentrations. NPCs were differentiated to mDPCs by midbrain fate specification using a combination of FGFb, FGF-8b, Shh, and ascorbic acid. The cells were maintained for 6 days in culture medium with the induction cocktail and concomitantly exposed to a therapeutically relevant dose of FLX (0.1 μM) or 0.1% DMSO (solvent control). Expression of TH was confirmed by immunocytochemistry (Fig. 1B) in control and FLX-treated cells and was higher in the FLX-treated group (44.13 ± 1.216) compared with control (34.28 ± 1.302) (Fig. 1A). However, the neuronal processes of TH+ cells in the FLX-treated group were smaller [mean neurite length (μm) 11.72 ± 0.5814] (Fig. 1C) and spindle shaped compared with well branched and longer processes in control group (19.37 ± 0.6112) (Fig. 1B, magnified region of interest), suggesting that FLX interferes with the differentiation process of dopaminergic cells.

To investigate underlying transcriptional changes, mDPCs were treated with FLX (1, 0.1, or 0.01 μM) and expression of genes critical for early regional specification and patterning of the ventral midbrain were measured. First, we analyzed the expression of the transcription factors orthodenticle homeobox 2 (Otx2) and homeobox engrailed-1 and 2 (En1 and En2). FLX treatment at 1 and 0.1 μM increased mRNA expression of Otx2 (1.24- and 1.21-fold change, respectively, P < 0.001, Fig. 2A), of En1 (1.17- and 1.19-fold change, respectively, P < 0.001, Fig. 2C) and of En2 (1.18-fold change, P = 0.015 and 1.23-fold change, P = 0.0003, Fig. 2D). In contrast, the expression of NK2 homeobox 2 (Nkx2.2), involved in midbrain fate determination and negatively regulated by Otx2, was downregulated by FLX treatment at all tested concentrations (P < 0.0001, Fig. 2B). Immunocytochemistry for En1 confirmed its higher expression in mDPCs following FLX treatment (25.34 ± 0.8686) compared with solvent control (17.40 ± 0.8201, n = 12) (Fig. 2E).

We further analyzed the expression of transcription factors essential for mDA neurogenesis, i.e., paired-homeodomain transcription factor 3 (Pitx3) and LIM homeobox transcription factor 1 α (Lmx1a). FLX treatment at all concentrations, except 1 μM FLX for Lmx1a, downregulated expression of both Pitx3 (0.89-fold change by 1 μM FLX, P = 0.0006; 0.05-fold change by 0.1 μM FLX, P < 0.001, and 0.14-fold change by 0.01 μM FLX, P < 0.001 respectively) and Lmx1a (0.76-fold change by 0.1 μM FLX, P = 0.0008 and 0.58-fold change by 0.01 μM FLX, P < 0.001) (Fig. 2G).
Next, we assessed whether FLX affects stemness by analyzing the expression of the neural stem cell marker Nestin (Nes) and the neuronal marker β-tubulin (Tubb3) in mDPCs. We found that Nes expression was increased (Fig. 3A) by FLX (1 μM FLX caused a 1.35-fold change, \( P < 0.001 \); FLX 0.1 μM caused a 1.50-fold change, \( P < 0.001 \) and FLX 0.01 μM caused a 1.23-fold change, \( P = 0.0010 \)) and Tubb3 (Fig. 3B) was decreased by lower concentrations of FLX (0.1 μM FLX induced a 0.79-fold change, \( P = 0.0028 \) and 0.01 μM FLX induced a 0.63-fold change, \( P < 0.0001 \)). Proper Notch-Hes signaling is essential for maintaining the neural progenitor identity and suppression of neuronal differentiation (Schuurmans and Guillemot, 2002). By analyzing the expression of Notch1 and its downstream effector Hes1, we observed a marked increase in the expression of both genes upon FLX treatment (Notch1: 2.40-fold change by 1 μM FLX, \( P < 0.0001 \); 1.67-fold change by 0.1 μM FLX, \( P < 0.0001 \); and 1.43-fold change by 0.01 μM FLX, \( P < 0.05 \). Hes1: 5.38-fold change by 1 μM FLX \( P < 0.0001 \); 2.51-fold change by 0.1 μM FLX, \( P < 0.001 \); and 1.91-fold change by 0.01 μM FLX, \( P < 0.05 \).) (Fig. 3, C and D). These data suggest that FLX interferes with signaling cascades underlying precursor cell maintenance and maturation.

**FLX Affects Expression of Genes Associated with Autism.** To evaluate if FLX modulates the expression of genes associated with ASD we measured the expression of forkhead box protein P2 (Foxp2), paired box 3 (Pax3), glutamate ionotropic receptor AMPA type subunit 2 (Gria2), retinoic acid receptor–related orphan receptor-α (Rora), and reelin (Reln), each of which not only plays a crucial role in midbrain patterning and neurogenesis (Nguyen et al., 2010; Sarachana et al., 2011; Lammert and Howell, 2016) but has also been implicated in autism (Borg et al., 2002; Nguyen et al., 2010; Bowers and Konopka, 2012; Uzunova et al., 2014; Lammert and Howell, 2016). We observed a downregulation of the expression of Pax3 (0.41-fold change by 1 μM FLX, 0.52-fold change by 0.1 μM FLX, \( P < 0.001 \)), Rora (0.60-fold change by 1 μM FLX, \( P < 0.05 \); 0.61-fold change by 0.1 μM FLX, \( P < 0.05 \); and 0.53-fold change by 0.01 μM FLX, \( P < 0.01 \)), and Reln (0.25-fold change by 1 μM FLX, \( P < 0.001 \); 0.43-fold change by 0.1 μM FLX, \( P < 0.0001 \); and 0.52-fold change by 0.01 μM FLX, \( P < 0.01 \). (Fig. 4). Additionally, increased levels of En1 and En2, as shown in Fig. 2, are also reported to correlate with ASD (Kuemmerle et al., 2007; James et al., 2014). These data suggest that FLX may indeed influence the expression of genes associated with ASD.

**FLX Affects ER Expression in Midbrain Precursors.** Since the mDPC cultures are too immature to form functioning synapses and any 5-HT produced is heavily diluted in the culture medium, the effect of FLX on the genes analyzed in this study is probably not due to 5-HT reuptake inhibition. Increasing evidence points to FLX having estrogen-disrupting effects (Foran et al., 2004; Menningen et al., 2008, 2010; Lister et al., 2009; Schultz et al., 2011; Lupu et al., 2017), and in view of our recent data showing that ERβ plays an important role in the differentiation of midbrain dopaminergic neurons in vitro (by maintaining proper Notch-Hes signaling, among others) (Varshney et al., 2017), we investigated by using ERβ knockout (BERKO) cells whether treatment with this SSRI affects the expression levels of ERs in WT mDPCs, as well as whether ERβ is involved in the observed effects of FLX.
We observed that both ER\textsubscript{\textalpha} and ER\textsubscript{\textbeta} are expressed in mDPCs (Fig. 5A) and that ER\textsubscript{\textalpha} mRNA expression was downregulated in both BERKO mDPCs (0.13-fold, \textit{P} < 0.001) and in WT mDPCs after FLX treatment compared with solvent control (0.24-, 0.20-, and 0.47-fold change by 1, 0.1, and 0.01 \textmu M FLX, respectively, \textit{P} < 0.001, Fig. 5B). Interestingly, in BERKO mDPCs, ER\textsubscript{\textalpha} levels were lower than in WT cells and FLX treatment did not reduce ER\textsubscript{\textalpha} expression any further compared with treatments in WT cells (Fig. 5B). ER\textsubscript{\textbeta} mRNA expression was also downregulated in WT mDPCs after FLX treatment compared with solvent control (0.69, 0.22-, and 0.39-fold change by 1, 0.1 and 0.01 \textmu M FLX, respectively, \textit{P} < 0.001, Fig. 5C).

**FLX Effects on Midbrain Fate Specification, But Not Maturation, Are Dependent on ER\textsubscript{\textbeta}**. To assess the role of ERs in mediating the effects of FLX on DA differentiation, we used BERKO NPCs to measure gene expression of the key factors analyzed above. We observed that in BERKO mDPCs, FLX had no or even opposite effects on the genes involved in mDPC specification (\textit{Otx2}, \textit{Nkx2.2}, \textit{En1}, and \textit{En2}) (Fig. 6) and on the stemness marker \textit{Nes} (Fig. 7A), suggesting that ERs are involved in the effects of FLX on early midbrain fate specification. In BERKO cells, \textit{Otx2}, \textit{En1}, \textit{En2}, and \textit{Nes} mRNA expression was downregulated after FLX treatment, compared with BERKO solvent controls (Fig. 6, A, C, and D; Fig. 7A). Likewise, FLX treatment of BERKO mDPCs did not affect expression of the autism-related genes \textit{Rora} or \textit{Reln}, which was 3-fold and 15-fold decreased, respectively, in BERKO compared with WT mDPCs in control-treated cells (Fig. 8).

On the other hand, in BERKO mDPCs FLX induced effects similar to those observed in WT mDPCs on the neuronal marker \textit{Tubb3} (0.90-fold change by 0.1 \textmu M FLX, \textit{P} = 0.0104; 0.80-fold change by 0.01 \textmu M FLX, \textit{P} < 0.0001, Fig. 7B) and on the neurogenesis factors \textit{Pitx3} (0.50-, 0.12-, and 0.12-fold change by 1, 0.1, and 0.01 \textmu M FLX, respectively, \textit{P} < 0.001) and \textit{Lmx1a} (0.75-fold change, \textit{P} < 0.001 for FLX 0.1 \textmu M; 0.53-fold change, \textit{P} < 0.001 for FLX 0.01 \textmu M), decreasing their mRNA expression compared with BERKO solvent controls (Fig. 7, C and D).

**FLX Is a Potential ER Binder.** As our results indicate the involvement of ERs in mediating some of the effects of FLX, we investigated if FLX could bind these receptors using molecular docking predictions. The results showed that both ER\textsubscript{\textalpha} and ER\textsubscript{\textbeta} can bind FLX through a combination of hydrophobic and polar interactions (Fig. 9). Hydrophobic interactions within ER ligand binding pockets were mainly mediated by leucine/isoleucine (Leu/Ile) and phenylalanine (Phe) amino acid residues, whereas hydrogen bonds could form between the secondary amino group present in FLX and glutamate (Glu353/Glu42) residues in ER ligand binding pockets. The low docking energies, located in the \textit{–}9.0 to \textit{–}8.5 bin for both ER\textsubscript{\textalpha} and ER\textsubscript{\textbeta} (Fig. 10), suggest that FLX has a fairly strong tendency to bind to ERs. The probabilities for binding of FLX to ER\textsubscript{\textalpha} and ER\textsubscript{\textbeta} are 0.88 and 0.74, respectively.

**Discussion**

To address prenatal FLX effects on the early differentiation of mDA fate from neural precursors, we employed a well
dopaminergic neurons (mDNs) have been successfully generated from mESCs, using highly controlled culture conditions to guide the differentiation process toward NPCs and, subsequently, to mDPCs and mDNs (Varshney et al., 2017). The mDNs obtained from both WT and BERKO mDPCs express the dopaminergic markers Th (tyrosine hydroxylase, the enzyme which catalyzes the rate-limiting step of catecholamine synthesis) and Slc6a3 (solute carrier family 6 member 3, the presynaptic dopamine transporter) and have functional dopamine synthesis and release (Varshney et al., 2017).

Our results show that FLX exposure, in therapeutically relevant doses, affects the differentiation process from NPCs to mDPCs. To our knowledge, there are no data on FLX kinetics in the fetus and FLX levels in the fetal brain. Thus we considered FLX concentrations as therapeutically relevant if they correspond to infant cord blood and plasma concentrations reported in the literature, which are in the range of 0.037–1.06 μM (Heikkinen et al., 2003; Kim et al., 2006; Gurnot et al., 2015).

We found that FLX upregulates transcription factors Otx2, En1, and En2, which are essential for ventral midbrain patterning, whereas Nkx2.2 was downregulated. Otx2, En1, and En2, among others, are expressed as early as embryonic days 7 to 8 (E7–8) in the mouse and are critical for the formation and positioning of the isthmic organizer, thus contributing to the patterning and determination of midbrain precursors (Hegarty et al., 2013; Arenas et al., 2015). Otx2 is also essential for the neurogenic potential of the floor plate (Hegarty et al., 2013). Conversely, Nkx2.2 is a negative regulator of midbrain dopaminergic development and is repressed by Otx2 (Abeliovich and Hammond, 2007). A lower expression of Nkx2.2 favors dopaminergic neuron differentiation at the expense of serotonergic neurons (Abeliovich and Hammond, 2007). Thus our results suggest that FLX increases differentiation of NPCs to midbrain precursors, which might lead to a decrease in serotonergic neurons.

On the other hand, we observed that the expression of the mDA markers Lmx1a and Pitx3 were downregulated upon FLX treatment. Lmx1a is induced by Otx2 and is required for the specification of mDA neurons and suppression of alternative fates (Hegarty et al., 2013; Arenas et al., 2015). Pitx3 is part of a network of transcription factors that cooperate to induce mDA neurogenesis and is also important for mDA neuronal survival (Hegarty et al., 2013). Concomitantly, the stemness marker Nes was upregulated, whereas the neuronal differentiation marker Tubb3 was downregulated. Interestingly, the mRNA expression of Pitx3, Lmx1a, and Tubb3 was lower after treatment with submicromolar FLX, suggesting that at 1 μM, FLX may be interacting with targets that compensate the downregulation of these neurogenesis markers. Th expression was increased following submicromolar FLX (0.1 μM); however, the nascent Th positive cells in this group had spindle-shaped morphology compared with more branched processes in untreated mDPCs. This could be attributable to feedback mechanisms during which cells produce more enzyme to synthesize dopamine, whereas the progenitors still cannot mature owing to aberrant signaling and expression of other factors such as reelin. Together, these findings suggest that although FLX promotes mDA fate determination, it prevents the midbrain precursors to progress into mature DA neurons.

These effects on dopaminergic differentiation might contribute to the association between developmental FLX...
exposure and increased risk for ASD. Importantly, some of the genes that we have found dysregulated by FLX are known to be associated with ASD (En1/2, Pax3, Rora, and Reln). En2 has been associated with ASD in genetic linkage studies, and increased En2 gene expression and protein levels were detected in the cerebellum of autistic subjects (Kuemerle et al., 2007; James et al., 2014). A study of postmortem brain tissue revealed decreased Rora protein levels in the cerebellum and frontal cortex of autistic individuals, which was consistent with increased CpG methylation in the upstream promoter region of Rora (Nguyen et al., 2010). Likewise, decreased Reln mRNA and protein was observed in the cerebellum and superior frontal cortex of autistic subjects (Lammert and Howell, 2016). Reln is critical for proper cell positioning and neuronal migration during development, and increasing evidence suggests that heterozygous mutations in Reln, including de novo mutations, contribute to the autistic phenotype (Lammert and Howell, 2016). Finally, a cryptic deletion including part of the Pax3 gene and a de novo translocation were observed in an ASD patient (Borg et al., 2002).

Several studies suggest that FLX affects estrogenic signaling in fish and mammals (Foran et al., 2004; Mennigen et al., 2008, 2010; Lister et al., 2009; Schultz et al., 2011; Lupu et al., 2017) and FLX has been shown to modulate ER transcriptional activation at (sub)micromolar concentrations using a reporter gene assay in breast cancer cells expressing both ERs (Pop et al., 2015). Proper estrogenic signaling is essential for normal brain development and influences apoptosis, synaptogenesis, and neuronal morphology (McCarthy, 2008). In a recent study, we have shown that ERβ facilitates midbrain dopaminergic fate and function largely ligand independently (Varshney et al., 2017) and that loss of ERβ had similar effects on DA differentiation, as we observe here with FLX treatment. Furthermore, ligand-mediated effects have also been shown as NPCs treated with estradiol during differentiation to mDPCs in vitro resulted in an increased proliferation of mDPCs expressing Lmx1a and in a higher proportion of dopaminergic neurons (Diaz et al., 2009). Estradiol action was mediated through nuclear ERs, as ICI182780 (ER antagonist) abolished the effect on proliferation of committed precursors and subsequent differentiation (Diaz et al., 2009). In our study, we showed that FLX decreases ERα and ERβ levels and that its effects on dopaminergic differentiation are abolished or even counteracted in BERKO cells, with the exception of Tubb3, Lmx1a, and Pitx3 downregulation. Notably, ERα is also strongly decreased in the BERKO cells and we show that FLX is predicted to bind to both ERs in silico, suggesting an involvement of one or both of these receptors in the observed effects. Nevertheless mDPCs do not form synapses, and although they express the serotonin transporter Slc6a4 (Supplementary Fig. 1, A and B), only low Tph2 levels are detected in these cells (Supplementary Fig. 1A), and any 5-HT produced is greatly diluted in the medium. Therefore, the effect of FLX in our mDPC model is probably not mediated through 5-HT reuptake inhibition, rather by its modulation of other factors, such as the ERs. Hence, our study suggests that the roles of the ER isoforms upon FLX administration should be further investigated.

For some of the genes examined (e.g., Pitx3, Nes, ERα, ERβ), we observed a non-monotonic dose-response (defined as a nonlinear relationship between dose and effect where the slope of the curve changes sign within the range of doses examined), and for other genes (e.g., Nkx2.2, Lmx1a, Tubb3) the effect size was greater at lower concentrations (in the nanomolar range) (Vandenberg et al., 2012). Both nonmonotonic and low-dose responses have been previously observed for chemicals that act on targets involved in endocrine regulation (including the ERs) and there is extensive literature from in vitro, in vivo, and epidemiologic studies in support of these concepts (Vandenberg et al., 2012).

Fig. 9. FLX in ERα (A) and ERβ (B) binding pockets represented via the Schrödinger Ligand Interaction Diagram.
In conclusion, we show here that FLX affects differentiation of dopaminergic neurons, possibly by interfering with the nuclear ERs. Our findings suggest that FLX increases neural specification toward the dopaminergic phenotype, yet decreases dopaminergic neurogenesis. This calls for further studies to establish if these molecular events are linked to the development of the dopaminergic system in humans and to address their involvement in the association between prenatal FLX exposure and increased ASD risk in children.

**Authorship Contributions**

*Participated in research design:* Lupu, Varshney, Inzunza, Nalvarte, Rüegg.
*Conducted experiments:* Lupu, Varshney, Mucs, Norinder, Nalvarte.
*Contributed new reagents or analytic tools:* Inzunza, Nalvarte.

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**Fig. 10.** Docking scores of ERα (A) and ERβ (B) with known active (light gray) and inactive (black) compounds in relation to FLX (dark gray).
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