Fluoxetine Affects Differentiation of Midbrain Dopaminergic Neurons In Vitro [S]

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ABSTRACT

Recent meta-analyses found an association between prenatal exposure to the antidepressant fluoxetine (FLX) and an increased risk of autism in children. This developmental disorder has been related to dysfunctions in the brains' rewards circuitry, which, in turn, has been linked to dysfunctions in dopaminergic (DA) signaling. The present study investigated if FLX affects processes involved in dopaminergic neuronal differentiation. Mouse neuronal precursors were differentiated into midbrain dopaminergic precursor cells (mDPCs) and concomitantly exposed to clinically relevant doses of FLX. Subsequently, dopaminergic precursors were evaluated for expression of differentiation and stemness markers using quantitative polymerase chain reaction. FLX treatment led to increases in early regional specification markers orthodenticle homeobox 2 (*Otx2*) and homeobox engrailed-1 and -2 (*En1* and *En2*). On the other hand, two transcription factors essential for midbrain dopaminergic (mDA) neurogenesis, LIM homeobox transcription factor 1 α (*Lmx1a*) and paired-like homeodomain transcription factor 3 (*Pitx3*) were downregulated by FLX treatment. The stemness marker nestin (*Nes*) was increased, whereas the neuronal differentiation marker β3-tubulin (*Tubb3*) decreased. Additionally, we observed that FLX modulates the expression of several genes associated with autism spectrum disorder and downregulates the estrogen receptors (ERs) α and β. Further investigations using ERβ knockout (BERKO) mDPCs showed that FLX had no or even opposite effects on several of the genes analyzed. These findings suggest that FLX affects differentiation of the dopaminergic system by increasing production of dopaminergic precursors, yet decreasing their maturation, partly via interference with the estrogen system.

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

ABBREVIATIONS: 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 engrailed-1; En2, homeobox engrailed-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 2; Otx2, orthodenticle homeobox 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.

TABLE 1
Primers used for mouse cDNA

	Forward	Reverse
36B4	GACACCCTCCAGGAAGCGA	GTGTTCGACAATGGCAGCAT
ERα	CTGTCCAGCAGTAACGAGAAAG	CACAGTAGCGAGTCTCCTTGG
ERβ	TGCCTGTAAACAGAGAGACCC	AGTCATTATGTCCTTGAATGCTTCT
Tubb3	TGTCATCCACCTTCATTGGC	AACATGGCTGTGAACTGCTC
Nes	AGGGCAAATCTGGGAACTTG	AGGGTTTCCATCTGCAAAGG
En1	ACAGAGAAAGCGAGCAAGAG	TTTTTCCCCCATAGCACCTG
En2	AGGTTCCAAGACGCTATCAC	TTTGAGCACTCCATCCAAGG
Otx2	GTCTTATCTAAAGCAACCGCCT	AGTAAATGTCGTCCTCTCCCT
Nkx2.2	GTGATCGTTGCCAAATGACG	TGCTCAGGAGACGAAAAAGG
Lmx1a	AGCGAGCCAAGATGAAGAAG	TTGTCTGAGCAGAAGTCAGC
Pitx3	ACGGCTCTCTGAAGAAGAAGC	CATGTCAGGGTAGCGATTCCTC
Th	ATTGGAGGCTGTGGTATTCG	ACTTTCAAAGCCCGAGACAG
Slc6a3	ATGTCTTCACACTGCTGGA	ATTGCTGGACACCGTAGAAC
Tph2	GTGACCCTGAATCCGCCTG	GGTGCCGTACATGAGGACT
Slc6a4	GTTGATGCTGCGGCTCAGATCT	GAAGCTCGTCATGCAGTTCACC
Foxp2	AAGGAGCAGTGTGGACTGTG	GCAGACTGGCATTTAGAGCTG
Gria2	GCCGAGGCGAAACGAATGA	CACTCTCGATGCCATATACGTTG
Pax3	CCGGGGCAGAATTACCCAC	GCCGTTGATAAATACTCCTCCG
Rora	GTGGAGACAAATCGTCAGGAAT	TGGTCCGATCAATCAAACAGTTC
Reln	TTACTCGCACCTTGCTGAAAT	CAGTTGCTGGTAGGAGTCAAAG

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

and social motivation following perinatal administration of SSRIs (fluoxetine and citalopram), which is suggestive of neurodevelopmental disorders with impaired social behaviors, 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
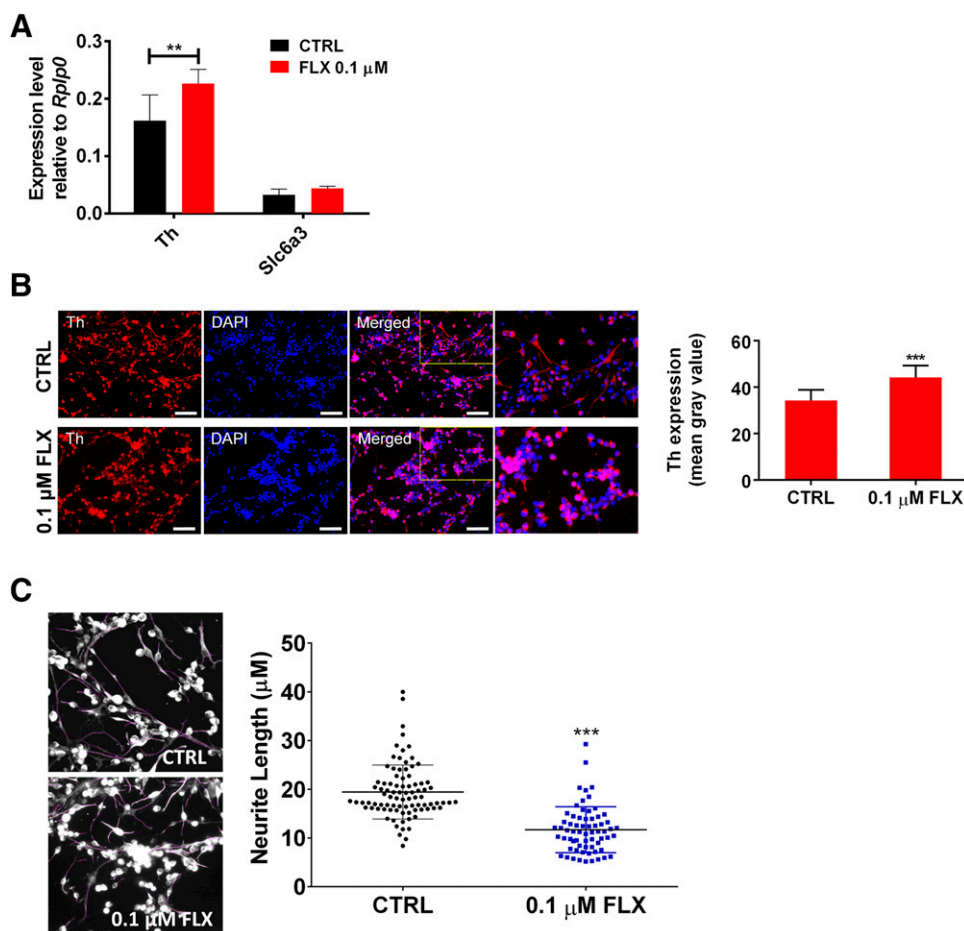
Fig. 1. Dopaminergic marker expression in mDPCs. (A) *Th* and *Slc6a3* mRNA expression relative to Rplp0 expression in mDPCs treated with solvent (CTRL) or with 0.1 μM FLX for 6 days. (B) Representative images of Th immunoreactivity in mDPCs upon control (CTRL) or 0.1 μM FLX treatments. Right panels 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).
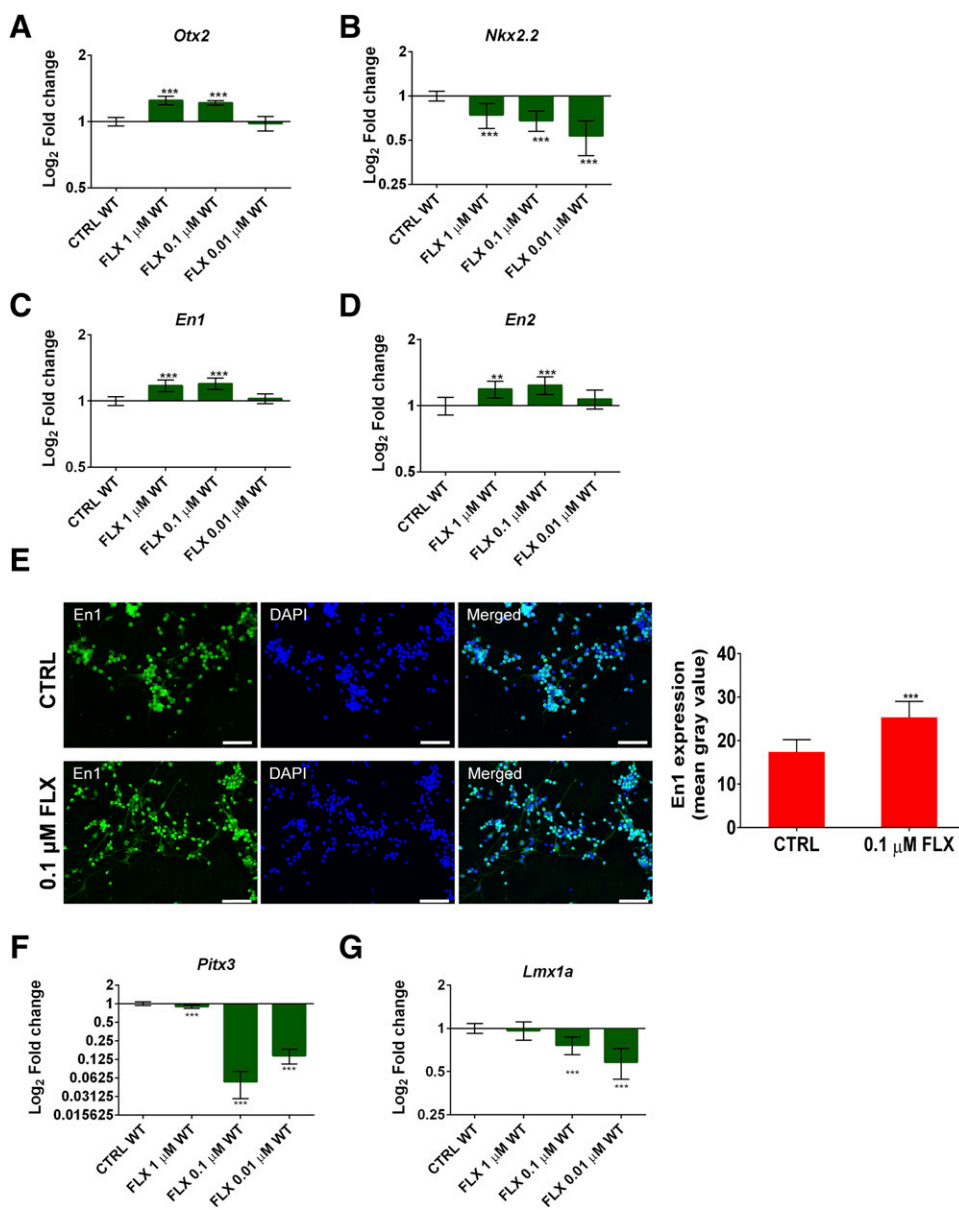
Fig. 2. FLX affects factors essential for midbrain patterning, specification, and mDA neurogenesis. WT NPCs were differentiated into mDPCs and concomitantly treated with 1, 0.1, and 0.01 μM FLX. Subsequently, mRNA expression of *Otx2* (A), *Nkx2.2* (B), *En1* (C), and *En2* (D) was measured using qPCR. (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 $\pm$ 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).

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; Pavăl, 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 mesocorticolimbic 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
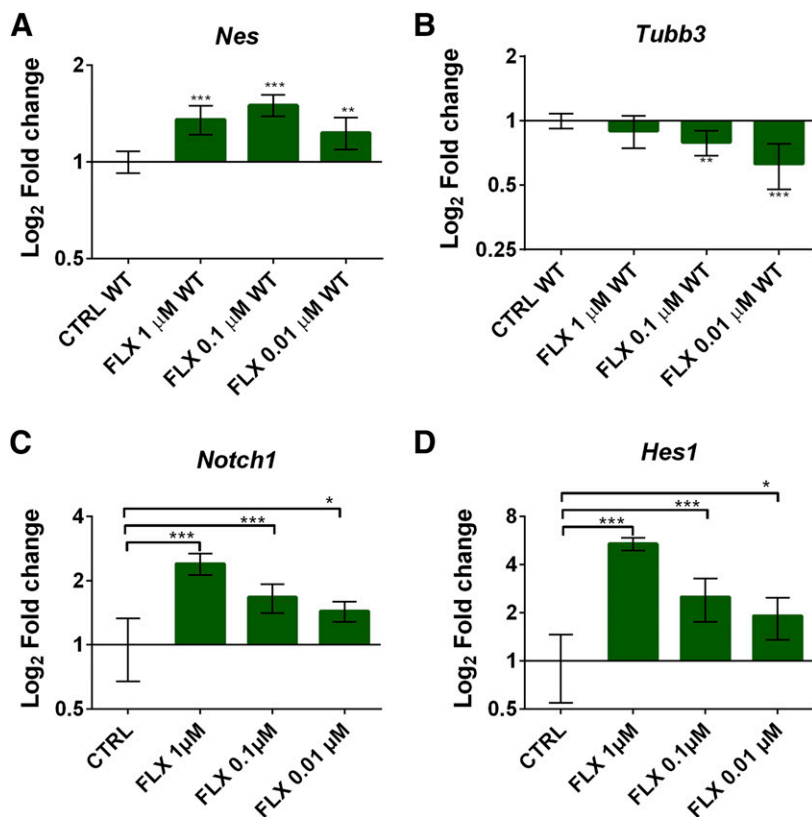
Fig. 3. FLX affects factors of neural stemness and differentiation markers in mDPCs. WT NPCs were differentiated into mDPCs and concomitantly treated with 1, 0.1, and 0.01 μM FLX. Subsequently, mRNA expression of *Nes* (A), *Tubb3* (B), *Notch1* (C), and *Hes1* (D) was measured using qPCR. Data represent means $\pm$ CI from three independent experiments in triplicates. Significant differences (one-way ANOVA) compared with control are marked with asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

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; Mennigen 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 NaHCO$_3$ (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 1×10^5 cells/cm^2. Media was changed every day and cells were passaged at confluency using Accutase (Life Technologies) for dissociation and spinning at 1500 rpm for 5 minutes.

Differentiation of NPCs to Midbrain Precursor Neurons. NPCs (WT and BERKO) were differentiated to midbrain dopaminergic precursor cell (mDPCs) by midbrain fate specification. In brief, NPCs were plated on poly-L-ornithine (50 μg/ml) and laminin (1 μg/ml)-coated dishes at a seeding density of 1.5–2×10^5 cells/cm^2 in neural expansion media supplemented with fibroblast growth factor-basic (FGFb, 10 ng/ml; PeproTech), fibroblast growth factor 8 basic

(FGF-8b, 100 ng/ml; Miltenyi Biotec GmbH, Teterow, Germany), Sonic Hedgehog (Shh, 400 ng/ml; PeproTech) and ascorbic acid (200 μm; Tocris, Bristol, UK). The culture media was also supplemented with experimental doses of FLX at 1, 0.1 and 0.01 μM (in DMSO) or DMSO at 0,1% (solvent control). Cells were maintained in this media for 6 days with a daily change of media. During this period NPCs generate midbrain precursor cells. On the 6th day media was removed and cells were washed with PBS and lysed with Buffer RLT (Qiagen, Hombrechtikon, Switzerland) supplemented with 1% β-mercaptoethanol.

Quantitative Real-Time PCR Analysis. Total RNA was isolated from mDPCs using the AllPrep DNA/RNA Mini Kit and treated with DNase I (both from Qiagen). RNA concentrations were measured using a NanoQuant plate on Tecan Infinite M200 (Tecan, Männedorf, Switzerland), and RNA quality was checked with Bioanalyzer (Agilent Technologies, Santa Clara, CA). Two thousand nanograms of RNA was used for reverse transcription with Superscript VILO cDNA Synthesis Kit (Life Technologies). Quantitative Real-Time polymerase chain reaction (qPCR) was performed using 5 ng of cDNA, self-designed (Primer 3.0 web) exon-exon spanning primers (see Table 1) and KAPA SYBR Fast Master Mix ABI Prism (Kapa Biosystems, Wilmington, MA), in a 7500 RealTime PCR System (Applied Biosystems, Foster City, CA). 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 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 compared with WT or BERKO controls were calculated using the Pfaffl or $\Delta\Delta$Ct 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 [phosphate-buffered saline (PBS),
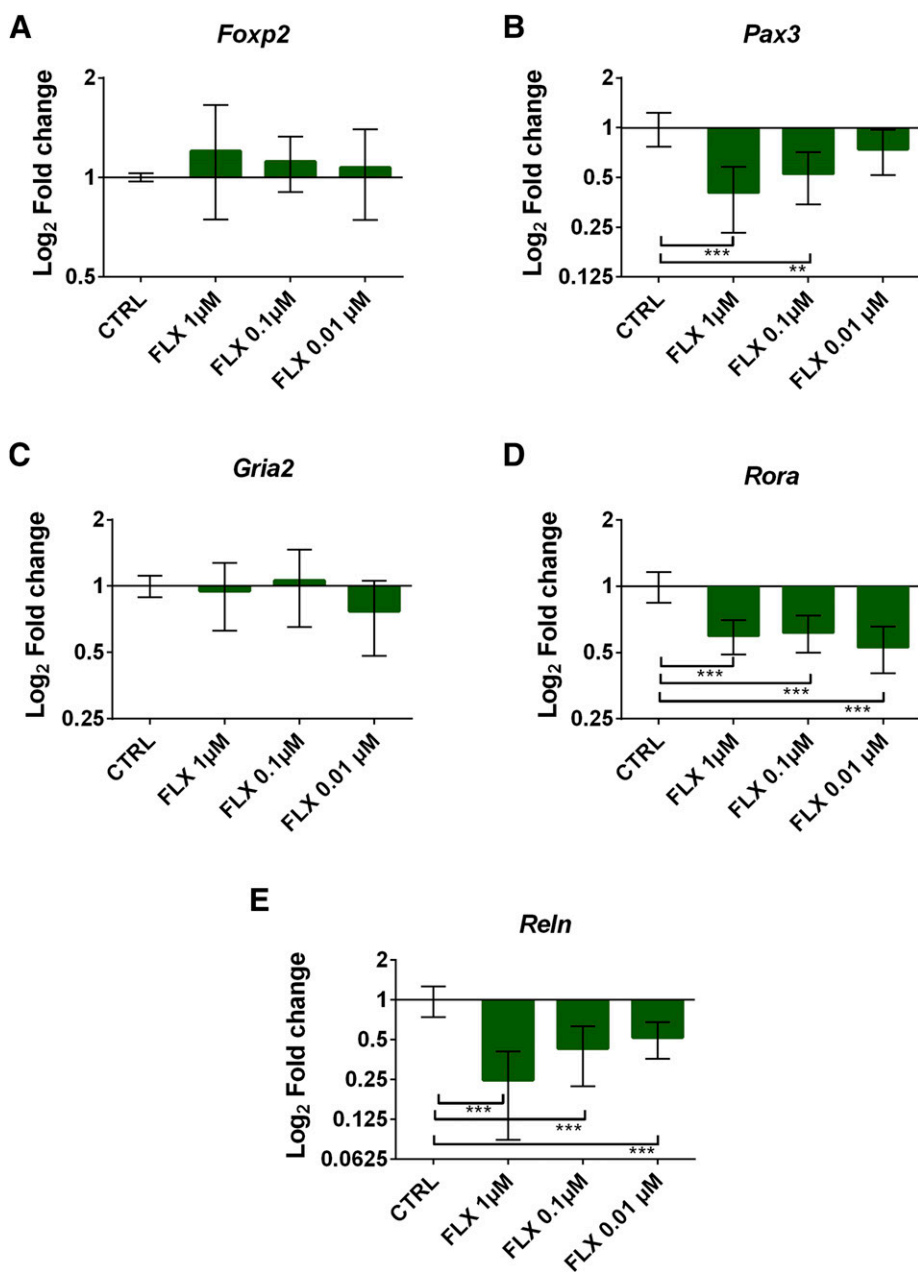
Fig. 4. FLX treatment of mDPCs affects some genes associated with autism. Relative change in mRNA expression levels of (A) *Foxp2*, (B) *Pax3*, (C) *Gria2*, (D) *Rora*, and (E) *Reln* for control (CTRL) or 1, 0.1, and 0.01 μM FLX treatments in mDPCs. Data represent means $\pm$ CI from three independent experiments in triplicates. Significant differences (one-way ANOVA) as compared to control are marked with asterisks (** for $P < 0.01$; *** for $P < 0.001$).

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× 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.

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-mean-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-like 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*
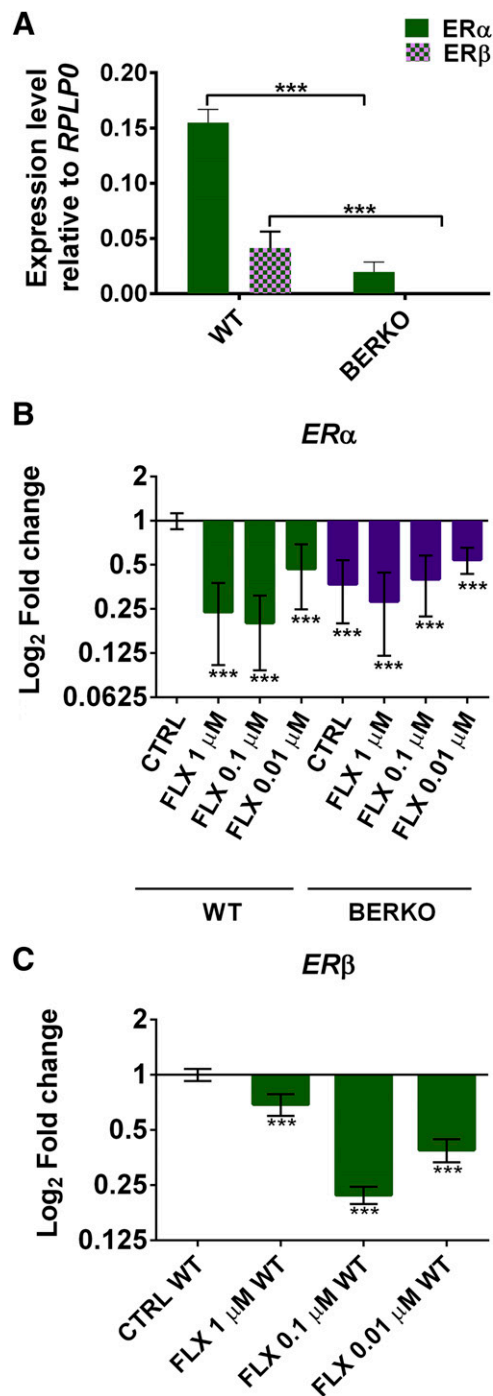
Fig. 5. FLX affects ERα and ERβ expression in mDPCs. WT and BERKO NPCs were differentiated into mDPCs and concomitantly treated with 1, 0.1, and 0.01 μM FLX. (A) mRNA expression of *ERα* and *ERβ* relative to *Rplp0* in WT and BERKO mDPCs, (B) fold change in mRNA expression of *ERα* in WT and BERKO mDPCs and (C) *ERβ* in WT mDPCs following FLX treatments. Data represent means ± CI from three independent experiments in triplicates. Significant differences (one-way ANOVA) compared with WT CTRL are marked with asterisks (***P < 0.001).

(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).
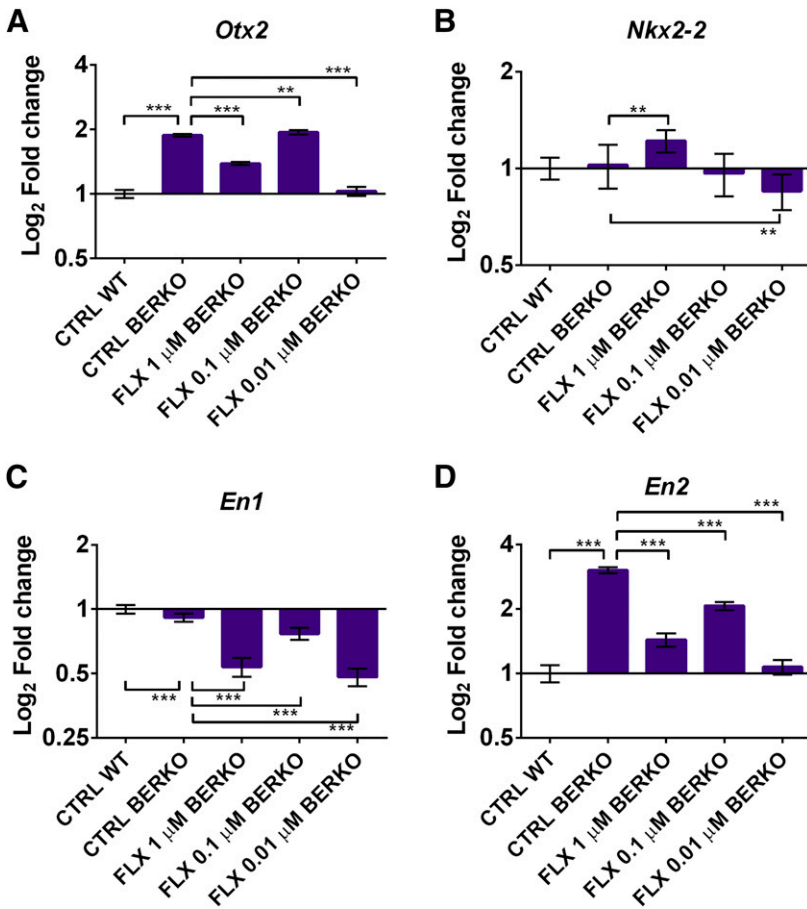
Fig. 6. Loss of ERβ attenuates the FLX-mediated effects on factors essential for midbrain patterning and mDPC specification. BERKO NPCs were differentiated into mDPCs and concomitantly treated with 1, 0.1, and 0.01 μM FLX. Subsequently, mRNA expression of *Otx2* (A), *Nkx2.2* (B), *En1* (C), and *En2* (D) was measured using qPCR. Data represent means ± CI from three independent experiments in triplicates. Significant differences (one-way ANOVA) are marked with asterisks (**$P < 0.01$; ***$P < 0.001$).

Next, we assessed whether FLX affects stemness by analyzing the expression of the neural stem cell marker Nestin (*Nes*) and the neuronal marker β3-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 (Kuemerle 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; Mennigen 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.
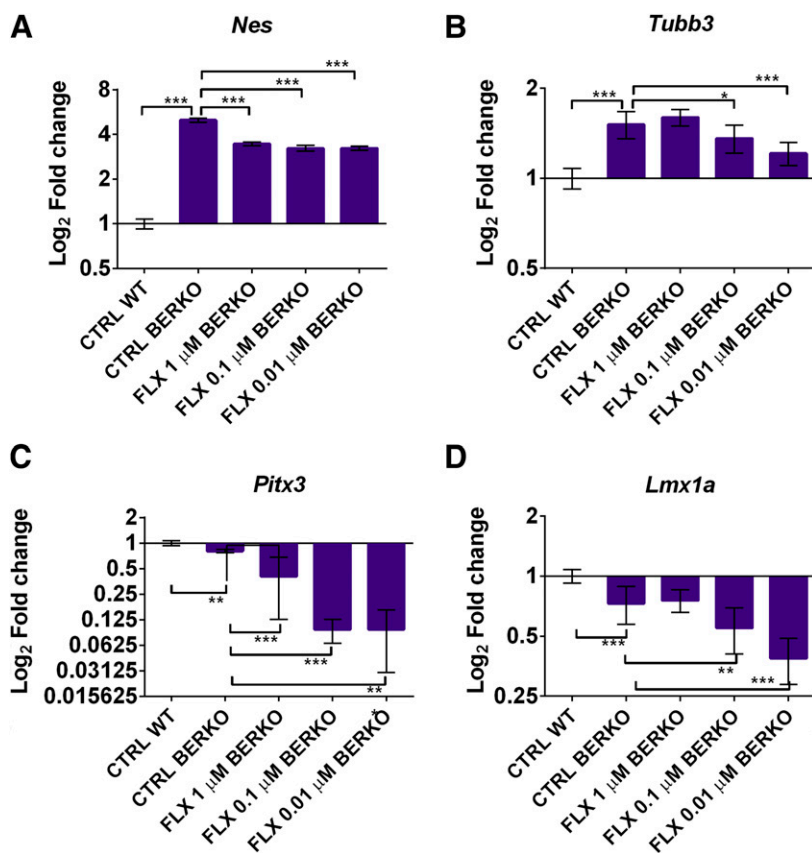
Fig. 7. FLX affects factors of neural stemness and mDA neurogenesis in mDPCs. BERKO NPCs were differentiated into mDPCs and concomitantly treated with 1, 0.1, and 0.01 μM FLX. Subsequently, mRNA expression of (A) *Nes*, (B) *Tubb3*, (C) *Pitx3*, and (D) *Lmx1a* was measured using qPCR. Data represent means $\pm$ CI from three independent experiments in triplicates. Significant differences (one-way ANOVA) are marked with asterisks (*$P < 0.05$, **$P < 0.01$; ***$P < 0.001$).

We observed that both ERα and ERβ are expressed in mDPCs (Fig. 5A) and that ERα mRNA expression was downregulated in both BERKO mDPCs (0.13-fold, $P < 0.001$) 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 μM FLX, respectively, $P < 0.001$, Fig. 5B). Interestingly, in BERKO mDPCs, ERα levels were lower than in WT cells and FLX treatment did not reduce ERα expression any further compared with treatments in WT cells (Fig. 5B). ERβ 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 μM FLX, respectively, $P < 0.001$, Fig. 5C).

FLX Effects on Midbrain Fate Specification, But Not Maturation, Are Dependent on ERβ. 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 (*Otx2*, *Nkx2.2*, *En1*, and *En2*) (Fig. 6) and on the stemness marker *Nes* (Fig. 7A), suggesting that ERs are involved in the effects of FLX on early midbrain fate specification. In BERKO cells, *Otx2*, *En1*, *En2*, and *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 *Rora* or *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 *Tubb3* (0.90-fold change by 0.1 μM FLX, $P = 0.0104$; 0.80-fold change by 0.01 μM FLX, $P < 0.0001$, Fig. 7B) and on the neurogenesis factors *Pitx3* (0.50-, 0.12-, and 0.12-fold change by 1, 0.1, and 0.01 μM FLX, respectively, $P < 0.001$) and *Lmx1a* (0.75-fold change, $P = 0.001$ for FLX 0.1 μM; 0.53-fold change, $P < 0.001$ for FLX 0.01 μM), 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 ERα and ERβ 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 -9.0 to -8.5 bin for both ERα and ERβ (Fig. 10), suggest that FLX has a fairly strong tendency to bind to ERs. The probabilities for binding of FLX to ERα and ERβ 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
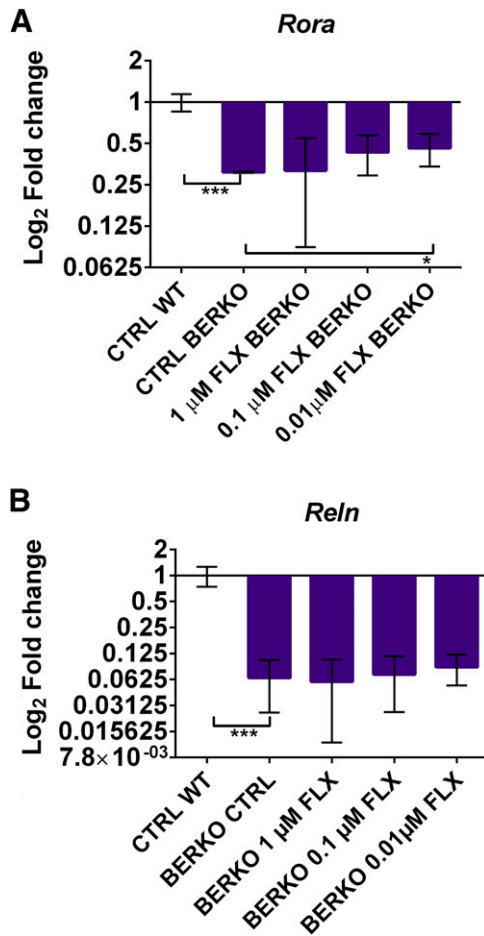
Fig. 8. FLX affects ASD-associated genes important for neuronal development. WT and BERKO NPCs were differentiated into mDPCs and concomitantly treated with 1, 0.1, and 0.01 μM FLX. Subsequently, mRNA expression of *Rora* (A) and *Reln* (B) was measured using qPCR. Data represent means ± CI from three independent experiments in triplicates. Significant differences (two-way ANOVA, followed by Turkey's test) are marked with asterisks (*$P < 0.05$; ***$P < 0.001$; ns, no significance).
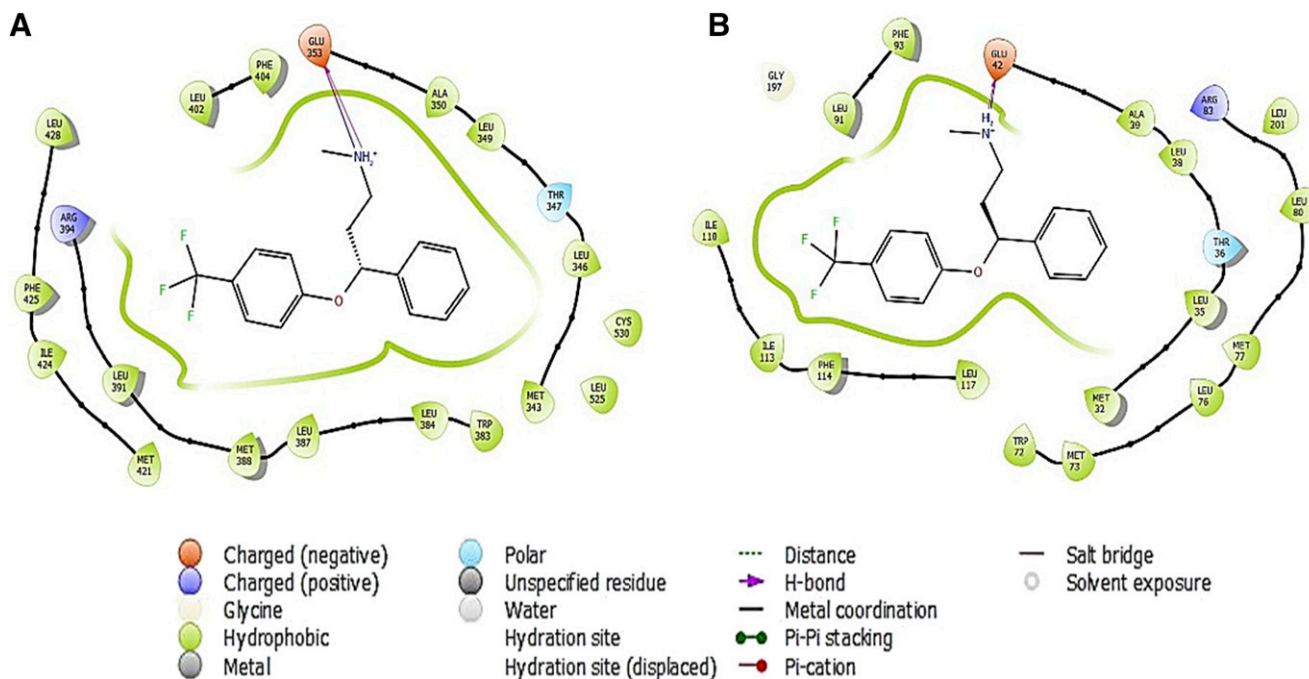
dopaminergic neurons (mDNs) have been successfully generated from mESCs, using highly controlled culture conditions to guide the differentiation process toward NPCs and, subsequently, to mDPCs and mDNs (Varshney et al., 2017). The mDNs obtained from both WT and BERKO mDPCs express the dopaminergic markers *Th* (tyrosine hydroxylase, the enzyme which catalyzes the rate-limiting step of cathecolamine 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

defined and highly controlled mESC cell model that reflects many of the key aspects of midbrain stem cell fate acquisition and development (Lee et al., 2000; Varshney et al., 2017). In vivo, the development of midbrain dopaminergic neurons begins with the patterning of the neural tube and is orchestrated by the isthmic organizer and the floor plate (Arenas et al., 2015). The isthmic organizer is located at the midbrain-hindbrain boundary and is represented by a group of cells with a complex pattern of gene expression that includes the signaling factors FGF8 (fibroblast growth factor 8) and Wnt1 (wingless-int1) and transcription factors *Otx2*, *Gbx2* (gastrulation brain homeobox 2), *Pax2* (paired box 2), and *En1/2*, among others (Arenas et al., 2015). The midbrain floor plate is a glial structure located along the ventral midline of the neural tube and modulates cell identity in the dorsal-ventral position by secreting the morphogens Shh and Wnt1 (Arenas et al., 2015). Thus, the coordinated activity of these transcription factors and morphogens regulates ventral midbrain patterning and the specification of ventral midbrain progenitor cells. Besides ventralization, the midbrain floor plate has neurogenic potential and is the source of dopaminergic precursors, subsequently giving rise to dopaminergic neurons (Hegarty et al., 2013). In vitro, mature midbrain

A

B

Fig. 9. FLX in ERα (A) and ERβ (B) binding pockets represented via the Schrödinger Ligand Interaction Diagram.

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 reveled 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 morphometry (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 (Díaz 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 (Díaz 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).

A

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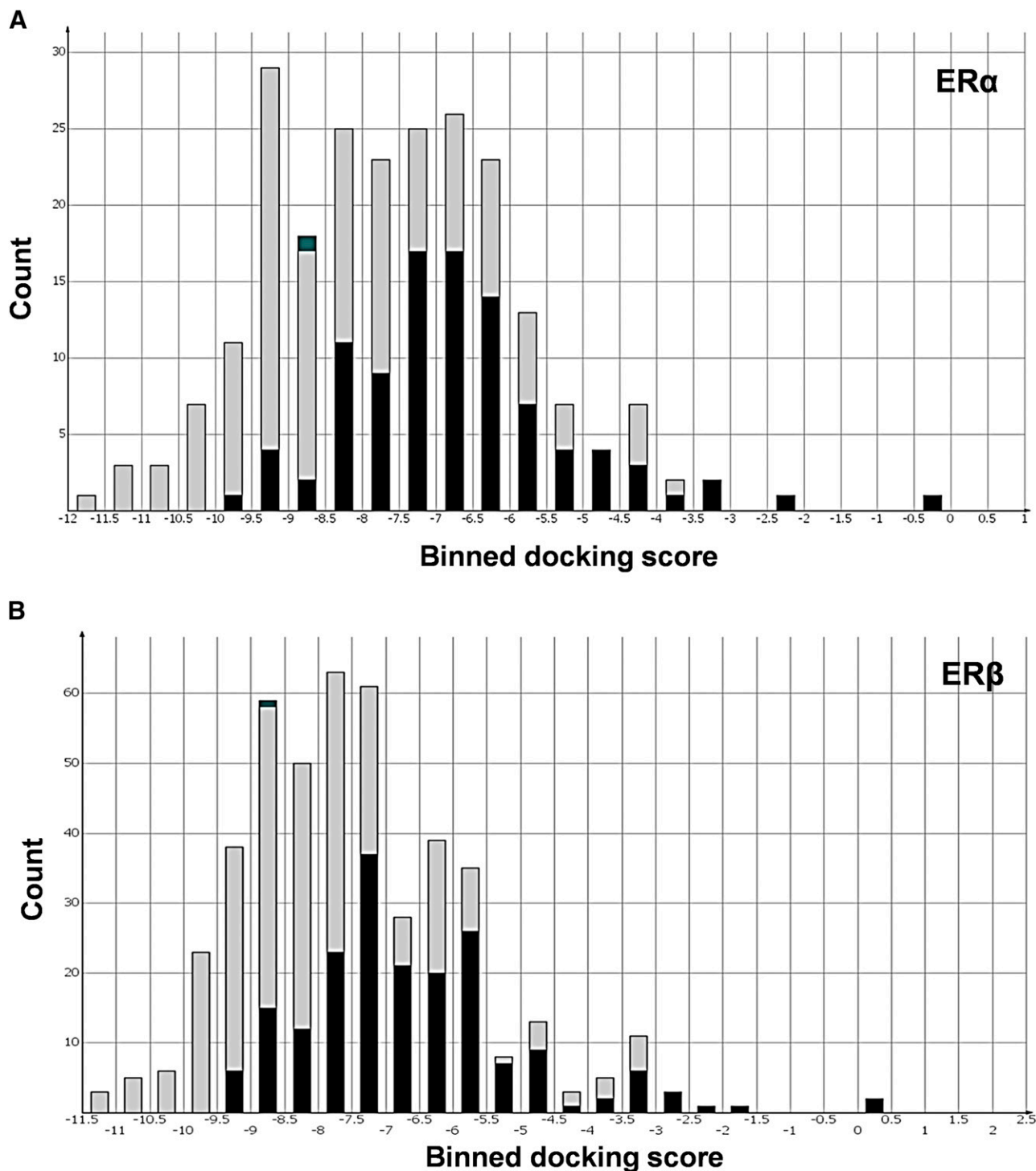
Fig. 10. Docking scores of ERα (A) and ERβ (B) with known active (light gray) and inactive (black) compounds in relation to FLX (dark gray).

Conclusion

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.

Performed data analysis: Lupu, Varshney, Mucs, Norinders, Nalvarte.

Wrote or contributed to the writing of the manuscript: Lupu, Varshney, Mucs, Inzunza, Norinder, Loghin, Nalvarte, Rüegg.

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