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Increased cellular turnover in response to fluoxetine in neuronal precursors derived from human embryonic stem cells

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ABSTRACT Previous reports have shown that antidepressants increase neuronal cell proliferation and enhance neuroplasticity both *in vivo* and *in vitro*. This study investigated the direct effects of one such antidepressant, fluoxetine , on cell proliferation and on the production of neurotrophic factors in neuronal precursors derived from human embryonic stem cells (hESCs; H9). Fluoxetine induced the differentiation of neuronal precursors, strongly enhancing neuronal characteristics. The rate of proliferation was higher in fluoxetine -treated cells than in control cells, as determined by MTT [3(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] assay. The CPDL (cumulative population doubling level) of the fluoxetine-treated cells was significantly increased in comparison to that of control cells (p<.001). Bromodeoxyuridine incorporation and staurosporine-induced apoptosis assays were elevated in fluoxetine-treated cells. Quantitative RT-PCR analysis revealed no significant differences in the expression of neurotrophic factors, brain-derived neurotrophic factor (BDNF); glial-derived neurotrophic factor (GDNF) and cAMP-responsive element-binding protein (CREB) between cells treated with fluoxetine for two weeks and their untreated counterparts. These results may help elucidate the mechanism of action of fluoxetine as a therapeutic drug for the treatment of depression. Data presented herein provide more evidence that, in addition to having a direct chemical effect on serotonin levels, fluoxetine can influence hESC-derived neuronal cells by increasing cell proliferation, while allowing them to maintain their neuronal characteristics.

KEY WORDS: *human embryonic stem cell, neuronal precursor, fluoxetine, cellular turnover*

Introduction

Human embryonic stem cells (hESCs), initially derived in 1998, show great potential as a source of cells for regenerative medicine and as a model for early human development (Thomson *et al.*, 1998). However, hESCs could have a more immediate use in pharmacotoxicology. To date, such an approach has primarily been used with mouse ESCs (mESCs). For example, Wobus and colleagues (Wobus *et al.*, 1991) generated differentiated cardiomyocytes from mESCs. These cells were then evaluated for chronotropic responses to adrenergic and cholinergic agents and to Ca^{2+} channel blockers. While the utilization of hESCs for pharmacotoxicology research would be quite valuable, there exist challenges associated with the standardization of culture conditions and methods (Wobus and Boheler, 2005). To this end, Dhara and colleagues (Dhara *et al.*, 2008) have recently shown the advantages of using a neural progenitor cell line grown without a feeder layer, similar to the one we used in this study. The aforementioned techniques can facilitate further study of molecular and biochemical mechanisms in early human neural differentiation and can potentially produce uniform neuronal cells for therapeutic use without concern about contamination from feeder

Abbreviations used in this paper: EB, embryoid body; EST, embryonic stem cell test; FLX, fluoxetine; HESCs, human embryonic stem cells; MEF; mouse embryonic fibroblast; NP, neuronal precursors.

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layers.

Depression is the most prevalent and costly mood disorder. It afflicts about 21% of the world population, and despite the multiple antidepressants available, not all patients respond to pharmacological treatment (Schechter *et al.*, 2005). While the use of postmortem human brain models in pharmacology and developmental toxicology has been developed, overall, human *in vitro* models to study the effects of drugs in treating mood disorders are scarce (Chen *et al.*, 2001; Ongur *et al.*, 1998; Rajkowska *et al.*, 1999). Current models have inherent limitations, such as limited availability of tissues and anatomical differences that, in turn, can affect the interpretation and repeatability of the results. Most *in vivo* studies of mood disorders have used laboratory animals, and while useful, they also have shortcomings, i.e., (1) genetic causes of mood disorders are difficult to trace in animals, (2) major primary symptoms of mood disorders are difficult to evaluate, and (3) interspecies differences between animal models (Nestler *et al.*, 2002).

Two mechanisms of depression have been hypothesized: chemical-based and network-based. Over the last few decades, most scientists and clinicians have accepted the premise that depression is caused by a chemical imbalance in the brain that can be corrected with medications. However, in the past decade, an alternative to simplistic chemical-based etiology has arisen; the network hypothesis suggests that depression reflects problems in information processing within neural networks in the brain (Castren, 2005). While intriguing, more research is needed to categorically support the network hypothesis.

Fluoxetine (FLX), a widely used, potent antidepressant commonly known as Prozac, was developed according to the chemical hypothesis. FLX is a selective serotonin reuptake inhibitor (SSRI) with a high selectivity for the 5-hydroxytryptamine transporter (5-HTT). It modulates synaptic serotonin concentrations in the central nervous system (CNS) (Wong *et al.*, 2005). Long-term administration of FLX can induce neural cell proliferation and neurogenesis in the hippocampus and prefrontal cortex of animal models both *in vivo* and *in vitro* (Chen *et al.*, 2006; Encinas *et al.*, 2006; Huang and Herbert, 2006; Kodama *et al.*, 2004; Malberg and Duman, 2003; Santarelli *et al.*, 2003). However, the mechanisms by which FLX promotes neural cell proliferation and neuroprotection *in vivo* are still unknown. Furthermore, the impact of FLX on the developing/prenatal CNS has not been determined, in part due to the lack of a laboratory model that can recapitulate human fetal development (Morrison *et al.*, 2005).

To examine the effects of FLX and the mechanisms of depression, hESCs with the potential to differentiate into"neuronal precursors can offer a useful alternative to the current *in vivo* models to study the effect of FLX specifically on early human embryonic development. In the present study, hESC-derived neuronal precursors were treated with FLX at a therapeutically relevant concentration and for the period required for clinical efficacy. During the treatment period, we evaluated the effects of FLX on cell proliferation, cell differentiation patterns, and expression of neurotrophic factors. The results presented here provide new evidence that FLX can indeed influence neuronal cells *in vitro*, supporting to the notion that an SSRI not only can correct chemical imbalances in the CNS but can modify its cellular architecture as well.

Results

Characteristics and differentiation of hESC-derived NPs after fluoxetine treatment

To avoid further potential contamination from feeder cells, which could alter accurate quantification of phenotypic markers during analysis, the differentiation of hESCs into neuronal precursors (NPs) was performed in the absence of any stromal cell line (Fig. 1). Generated hESC-derived NP cells showed a stable, normal karyotype (46, XX) (Suppl. Fig. 2).

Immunofluorescence and RT-PCR analyses demonstrated that the hESC-derived NPs were strongly positive for nestin, PSA-NCAM, and β-tubulin. Neuronal differentiation was assessed by determining the number of cells expressing neuronal markers as a percentage of total cells counted in a selected area. The percentage of nestin-positive($^+$) cells was 88.2 ± 3.35%, while percentages for PSA-NCAM$^+$, βIII-tubulin$^+$, MAP2$^+$, and serotonin$^+$ were 67.9% ± 3.04%, 78.5% ± 6.45%, 50.6% ± 11.5%, and 29.3% ± 4.95%, respectively. A lower percentage of cells expressed GFAP, CK-8, RIP, and TH, with percentages of 5.5% ± 3.2%, 3.1% ± 2.7%, 2.7% ± 1.5%, and 1.3% ± 0.8%, respectively (Fig. 3A). None of the cells expressed Oct4 (data not shown). Therefore, the hESC-derived NP cells expressed the character-

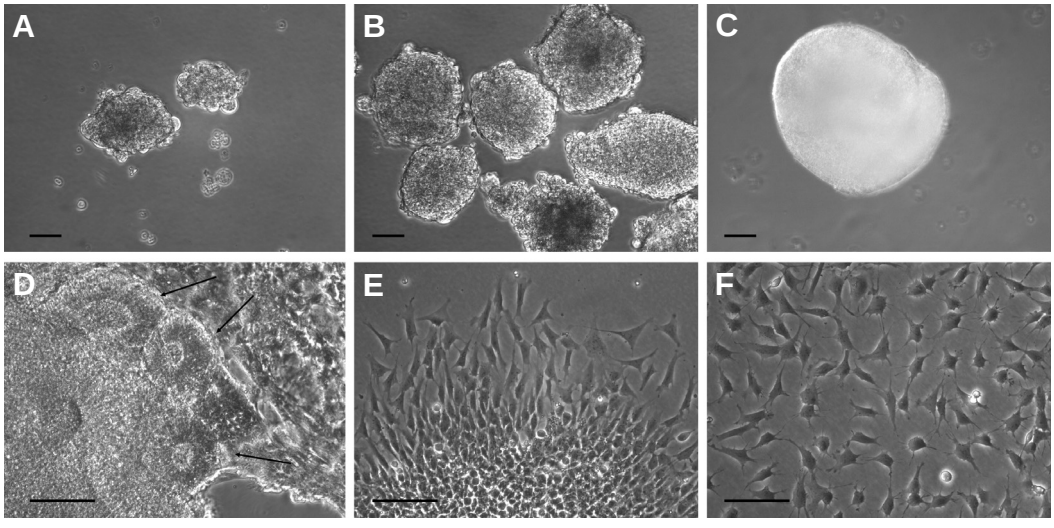


Fig. 1. Differentiation and isolation of hESC-derived neuronal precursors (NPs). (A) *hESC clumps floating on day 9.* **(B)** *Embryoid bodies (EBs) grown in DMEM/F12 with N2 supplementation for 8 days.* **(C)** *By day 18, EBs were clearly demarcated with a smooth surface.* **(D)** *After attachment, EB formed neuronal rosettes (arrow).* **(E)** *NPs migrating from the periphery of neuronal rosettes.* **(F)** *Dissociated single NPs were propagated as attached monolayer cultures. Scale bars A,B,C, 50 μm; D,E,F, 1 mm.*

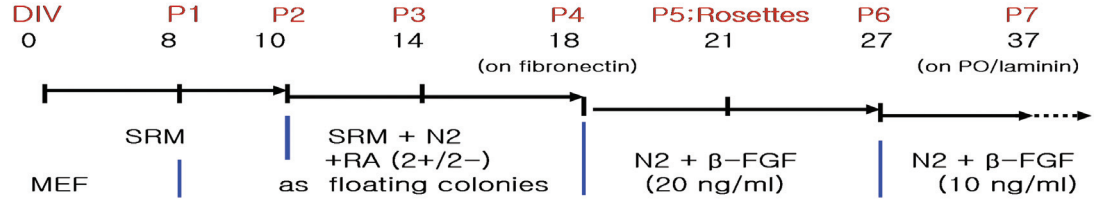


Fig. 2. Schematic representation shows culture conditions and protocol of neuronal differentiation. *hESCs were plated on MEF feeders for eight days. hESC colonies were mechanically isolated and replated in feeder-free dishes as floating colonies (embryoid bodies; EBs) until day ten. EBs were attached on fibronectin-coated plates. Neural rosettes formed on days 21-27, were mechanically isolated and replated on poly-ornithine/laminin-coated plates. Neuronal precursors derived from neural rosettes were propagated in the presence of FGF-2 as attached monolayer cultures and passaged weekly.*

istic markers of early neuronal cells. The fact that only a small percentage of cells were immunoreactive to RIP and CK-8 antibodies suggests that glial and epithelial cells were the least abundant types of cell in the cultures. The cells used in this study are fully capable of giving rise to astroglia, oligodendrocytes and neurons (data not shown) however, our goal in this differentiation protocol was to induce the NPs specifically into neuronal lineage. Semiquantitative RT-PCR analysis also showed results similar to the immunofluorescence data (Fig. 3B).

Following two weeks of culture with FLX, RT-PCR and immunofluorescence analyses showed that NPs expressed significantly more neuronal markers than control cells and continued to be negative for glial markers (Fig. 4A, 4B). No differences in the expression of MAP2 and serotonin were observed between control cells and those treated with FLX (Fig. 4A). However, the expressions of nestin detected by RT-PCR in control and FLX-treated cells are lower than Immunofluorescence analyses, This might be due to different transcriptional regulation and protein turn-over rates (Di Lieto *et al.*, 2007).

Cell growth pattern of fluoxetine -treated NPs, with or without FGF-2

This study initially evaluated the influence of FGF-2 on FLX-treated cells.

NPs were cultured under four factorial combination of 4 ng/ml FGF-2 and 0.5 μg/ml FLX (FLX-/FGF-2+; Suppl Fig. 3-A1, FLX+/FGF-2+; Suppl Fig. 3-A2, FLX-/FGF-2-; Suppl Fig. 3B-, and FLX+/FGF-2-; Suppl Fig.3B-2). In the absence of FGF-2, NPs treated with FLX showed a clear difference in their morphology and growth pattern compared to those of non-FLX treated cells in two weeks. Numerous cell aggregates, similar to neurospheres were formed in FLX treated cells as opposed to scattered individual cells of control NPs (Suppl Fig. 3-B1,B2). When FGF-2 was present in the media, FLX treated and non-FLX treated cells were similar in their morphological characteristics with the appearance of large cell clumps, regardless of FLX treatment (Suppl Fig. 3-A1,A2). Due to the masking effect of FGF-2 on FLX treatment we observed, subsequent experiments were conducted without FGF-2 treatment.

Effects of fluoxetine on cell proliferation and death in neuronal precursors

The effects of FLX concentration on cell proliferation rates were determined using an MTT assay. When 10 μg/ml of FLX was used, 50% of NPs died within 24 hrs of exposure to the compound. When the dosage was reduced to 1 μg/ml of FLX, proliferation rates were similar to those of the control and of cells treated with 0.5 μg/ml. However, 50% of cells died after fourteen days of treatment with 1 μg/ml. On the other hand, NPs survived for up to fourteen days in less than 0.5 μg/ml of FLX, as did the control cells. An FLX concentration of less than 0.5 μg/ml is within the therapeutic range for FLX in adult human plasma, indicating that FLX has no cytotoxic effects as clinically used (Suppl Fig. 4). Briefly, the MTT assay showed that the proliferation rate of FLX-treated cells

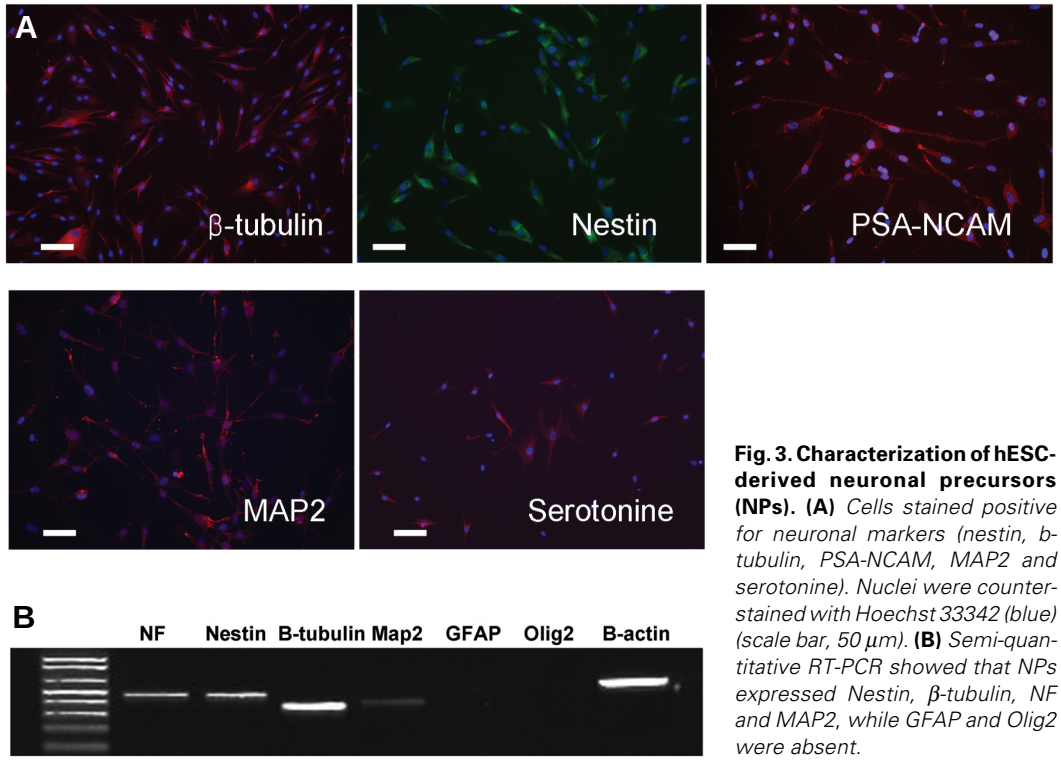


Fig. 3. Characterization of hESC-derived neuronal precursors (NPs). (A) *Cells stained positive for neuronal markers (nestin, b-tubulin, PSA-NCAM, MAP2 and serotonine). Nuclei were counterstained with Hoechst 33342 (blue) (scale bar, 50 μm).* **(B)** *Semi-quantitative RT-PCR showed that NPs expressed Nestin, β-tubulin, NF and MAP2, while GFAP and Olig2 were absent.*

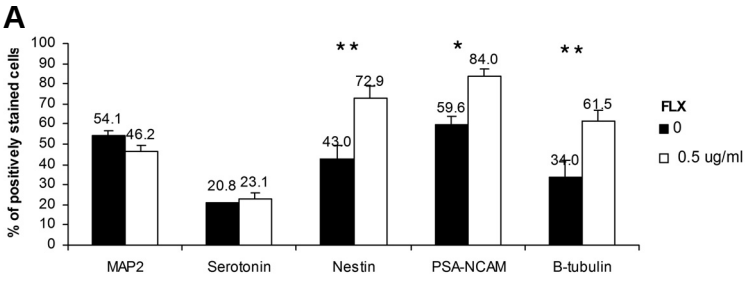


Fig. 4. Characteristics of hESC-derived neuronal precursors (NPs) changed after 2 weeks of fluoxetine treatment. (A) *Immunostaining showed that rates of Nestin, PSA-NCAM and β-tubulin positive staining significantly increased compared to that in control NPs (** = p<.01, * = p<.05). Rates of MAP2 and Serotonin positive NPs did not change.* **(B)** *RT-PCR showed that expression of Nestin, NF and β-tubulin were more increased in fluoxetine(FLX)-treated than in control cells. Fluoxetine-treated cells continued to be negative for glial markers (GFAP and Olig2). No difference in expression of MAP2 or serotonin was observed between control cells and those treated with fluoxetine.*

increased more than those of control cells at less than 0.5 µg/ml of FLX during the first week, despite showing no statistically significant difference between different concentrations of FLX-treated NPs in overall proliferation rates.

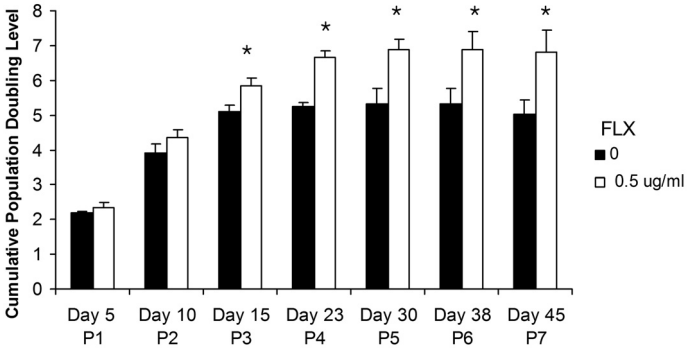
CPDL was measured with and without FLX to determine the long-term proliferative potential of FLX-treated NPs. After 45 days of continuous passaging, totals of 6.81 and 5.03 CPDLs were found in FLX-treated and control cells, respectively. When the cells were passaged at regular intervals (every five to seven days), the CPDL of FLX-treated NPs was significantly increased at day 15 ($p<.001$; Fig. 5).

To examine the influence of FLX on DNA synthesis, the number of BrdU-positive NPs was counted by immunostaining at days 0, 3, and 7 after FLX treatment (0.5 µg/ml). The percentage of BrdU-positive cells at days three (54.9% ± 0.3%,) and seven (55.4% ± 0.4%) was significantly increased in FLX-treated cells compared to controls (Fig. 6, Table 1).

The rate of apoptosis induced by staurosporine (STS) in FLX-treated NPs was measured using DNA fluorochrome Hoechst staining. Upon exposure of the control cells to STS (200 nM) for 20 hrs, about 7.35% of the cells died at day 0. When NPs were treated with 0.5 µg/ml FLX, the percentage of apoptotic cells was 22.03% at day three, 31.25% at day seven, and 51.62% at day 14. Using 1.0 µg/ml FLX, the percentage of apoptotic cells was 25.18% at day three, 60.59% at day seven, and 62.86% at day 14. These data indicate that apoptosis increases in NPs in the presence of FLX when the concentration and length of exposure increase compared to controls ($p<.05$ and $p<.001$, respectively; Fig. 7). It appears that FLX treatment itself does not promotes apoptosis but manifests the apoptosis induced by STS. The role of FLX in apoptotic gene expression (Bcl-2, Bcl-xL, and Bax) in NPs was also investigated by real time RT-PCR analysis two weeks after the onset of FLX treatment. The expression of these genes showed no statistically significant differences between FLX-treated and control cells (data not shown).

Effects of fluoxetine on production of neurotrophic factors

Considering that changes in the levels of neurotrophic factors can indicate neuronal plasticity in the brain (Benninghoff *et al.*, 2002; Castren *et al.*, 2007; Nibuya *et al.*, 1996), we evaluated changes in the production of brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), cAMP-responsive element-binding protein (CREB), 5-hydroxytryptamine receptor 1A (5HTR1A), 5-hydroxytryptamine receptor 2A (5HTR2A) and 5-hydroxytryptamine transporter (5HTT), as measured by real-time RT-PCR analysis after two weeks of FLX treatment. We also measured protein expression of BDNF, CREB, p-CREB (phosphorylated CREB) and 5HTT by western blotting after two



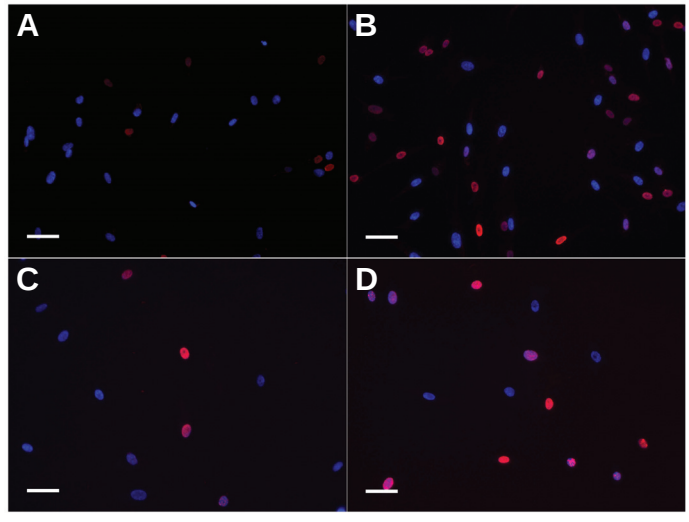


Fig. 5 (Left). Cell proliferation assays for hESC-derived neuronal precursors (NPs) with and without fluoxetine. *Cumulative population doubling level (CPDL) of NPs cultured with and without fluoxetine (FLX). CPDL of FLX-treated cells were significantly increased from day 15 until day 45 (* = p<.001).*

Fig. 6 (Right). Immunofluorescent BrdU-incorporation of neuronal precursors (NPs). *Percentage of BrdU-positive cells (red) was significantly increased in hESC-derived NPs cultured with fluoxetine (0.5 µg/ml) for 3 days* **(B)** *and 7 days* **(D)** *(p<.05 and p<.01, respectively), compared with control NPs cultured without fluoxetine for 3 and 7 days* **(A,C)**. *Scale bar, 50 µm.*

TABLE 1

PERCENTAGE OF BrdU-IMMUNOPOSITIVE NEURONAL PRECURSORS, WITH AND WITHOUT FLUOXETINE, OVER TIME

	Time of drug treatment (% of BrdU-labeled cells)		
Drug conc. (μg/ml)	Day 0	Day 3	Day 7
0	41.4 ± 0.2	40.1 ± 0.1	33.0 ± 0.2
0.5	42.1 ± 0.4	54.9 ± 0.3*	55.4 ± 0.4**

(*; p<.05, **; p<.01)

weeks of FLX treatment. We found no statistically significant differences in gene and protein expression between the FLX-treated cells and controls (data not shown).

Discussion

Drug development presents numerous challenges beyond the demonstration of efficacy. Of great concern is the inability to anticipate toxicity in specific tissues, as well as potential teratogenicity. While numerous animal models can help predict the risks of drugs before the initiation of clinical trials, there is a tremendous need for human *in vitro* models that can be used to evaluate toxicity early in development.

Over the past ten years, ECVAM (the European Center for the Validation of Alternative Methods) has conducted several *in vitro* embryotoxicity tests — such as the whole-embryo culture (WEC) test, the rat limb bud micromass (MM) test, and the embryonic stem cell test (EST) — to evaluate chemicals known to have embryotoxic potential *in vivo.* These tests also very effectively reduce the number of *in vivo* animal experiments conducted (Genschow *et al.*, 2002; Scholz *et al.*, 1999). The most useful embryotoxicity test is the EST, which uses two well-established cell lines: a pluripotent mouse embryonic stem cell (mESC) line (D3) and differentiated 3T3 fibroblasts (Scholz *et al*., 1999). EST studies take advantage of the potential of mESCs to detect embryotoxicity *in vitro*. However, *in vitro* tests using mESCs have limitations due to species-specific developmental differences (Pouton and Haynes, 2007). Accordingly, hESCs can provide a more useful model to test new therapeutic compounds and also can be used as an *in vitro* model to test whether a specific drug promotes or interferes with lineage specific differentiation program.

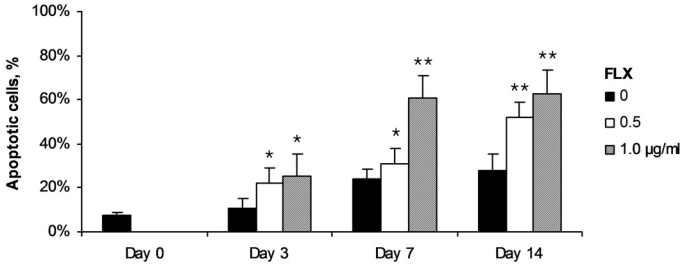


Fig. 7. Staurosporine-induced apoptosis in neuronal precursors (NPs). *Fluoxetine (FLX)-treated cells were exposed to 200 nM staurosporine (STS) for 20 h. Apoptosis was evaluated by Hoechst 33342 staining. Fragmented nuclei with condensed chromatin, which reliably indicate apoptosis were counted. Since FLX and STS treatment were started simultaneously, only one apoptotic index is plotted at D0. Data are presented as mean ± SE (%). (*; p<.05, **; p<.001).*

FLX, currently one of the most widely prescribed antidepressant medications. While it is known that FLX targets early progenitor cells in the adult brain (Encinas *et al.*, 2006), its effect upon the developing brain has not been completely determined, in part due to the lack of a proper human model. In an effort to identify the therapeutic and toxic impacts of FLX *in vivo,* we describe an experimentally novel approach to examining various aspects of FLX's effects on NPs derived from hESCs.

The results showed that FLX-treated NPs proliferated within one week after the addition of the drug and to a greater degree than untreated cells. This indicates that the mitogenic activity induced by FLX occurred during the early phases of these cultures. Our findings differ from previous studies, which argued that cell proliferation occurred after two or three weeks of FLX treatment *in vivo* or *in vitro* (Kodama *et al.*, 2004; Malberg and Duman, 2003; Manev *et al.*, 2001; Santarelli *et al.*, 2003). The current study, however, produced results similar to those of Chen *et al.,*2007), who observed cell proliferation under *in vitro* conditions after seven days, though they used different cell type and culture conditions. It is highly likely that the discrepancies observed are due to differences in the experimental design — i.e., *in vivo* or *in vitro* tests.

The MTT assay and CPDL data demonstrated that there was a different proliferative effect between the two tests observed in the cells, depending primarily on the different cell culture methods *in vitro*. For the MTT assay, cells were not passaged. However, for the CPDL analysis, NPs were passaged at regular intervals, and they survived for up to 45 days. Control cells plateau their proliferation rate from Day 15 onward, while FLX-treated cells plateau on their proliferation on Day 23. We conclude that the effect on proliferation rate was higher in FLX-treated cell, as measured by CPDL. The results of the MTT assay showed that FLX has no cytotoxic effects at less than therapeutic concentrations (<0.5 μg/ml); however, this does not rule out the possibility of any teratogenic effect.

In the present study, the results of the STS-induced apoptosis assay were affected by the concentrations and time courses of the administration of FLX. Apoptosis increased as the concentrations and/or time increased, but no changes in apoptotic gene expression (Bcl-2, Bcl-xl, and Bax). This indicates that although induced apoptosis is usually associated with changes in the amount of apoptotic gene transcripts, induced without changes in the transcript level of these genes probably due to posttranscriptional induction/regulation of the pathway. Previous studies examined the effects of FLX on apoptosis with different types of cell, such as adult neurons, hepatocytes, lymphoma cells, and neuroblastoma cells (Chen *et al.*, 2007; Chiou *et al.*, 2006; Koch *et al.*, 2003; Lee *et al.*, 2001; Levkovitz *et al.*, 2005; Nahon *et al.*, 2005; Serafeim *et al.*, 2003). However, these studies used nonhuman CNS cells and animal models. Previous reports have shown conflicting evidence regarding the effect of FLX on apoptosis. Some studies showed an increase in apoptotic activity due to FLX in different cell lines using various methods (Koch *et al.*, 2003; Levkovitz *et al.*, 2005; Serafeim *et al.*, 2003), while others found an increase in antiapoptotic activity (Chen *et al.*, 2007; Chiou *et al.*, 2006; Lee *et al.*, 2001; Nahon *et al.*, 2005). These conflicting findings may have resulted from differences in cell lines, FLX concentrations, or the duration of treatment. Our results, similar to those reported

by others (Koch *et al.*, 2003; Levkovitz *et al.*, 2005; Serafeim *et al.*, 2003), detected an increase in apoptotic activity within one week of FLX treatment.

Interestingly,"FLX treatment increased in both cell proliferation and induced apoptotic activity in our experiments, consistent with the findings in the study by Sairanen *et al.* (2005) (Sairanen *et al.*, 2005), which was conducted using an *in vivo* animal model after ten and 20 days of FLX treatment. We provide further evidence that FLX may activate cell proliferation and augment the effect of apoptotic agents in hESC-derived NPs.

The observed cellular characteristics and differentiation patterns demonstrate that FLX enhanced the neuronal rather than the glial properties. Markers increased by FLX were early neuronal markers, such as nestin, PSA-NCAM and β-tubulin. PSA-NCAM-induced expression by FLX is of interest since it has been described as a gene involved in neurogenesis (Kiss and Rougon, 1997; Varea *et al.*, 2006). In addition, PSA-NCAM is closely associated with p-CREB involved in neuroplasticity and cell survival (Nakagawa *et al.*, 2002). Therefore, the pattern of PSA-NCAM expression and cell proliferation observed in this study supports the hypothesis that one mechanism of action of FLX is via neurogenesis, providing further evidence that the mechanism of depression is associated with abnormal processing of the neural network.

Until recently, studies of depression and antidepressants have focused on intracellular pathways, which are known to be activated by a number of extracellular signals, such as growth factors, stress, and neurotransmitters (Malberg, 2004; Malberg and Blendy, 2005). These signal pathways regulate various cellular processes associated with neuroplasticity and neurogenesis. The transcription factors CREB and BDNF are the targets of diverse types of antidepressants (Blendy, 2006; De Foubert *et al.*, 2004; Josselyn and Nguyen, 2005). Chronic FLX administration promotes neurogenesis and synaptogenesis in the adult hippocampus (Grote *et al.*, 2005; Malberg *et al.*, 2000), as well as increased expression of the neurotrophins BDNF and GDNF (Hisaoka *et al.*, 2007; Nibuya *et al.*, 1995). These molecular and cellular events seem necessary to mediate the therapeutic effects of FLX. However, there are conflicting reports on the effect of FLX on CREB and BDNF. Some studies have observed increases in CREB and BDNF following *in vivo* FLX administration, and the authors attributed this increase to the type of anatomical brain lesions and to their experimental designs (Chen *et al.*, 2001; Malberg and Blendy, 2005; Nibuya *et al.*, 1995). Other studies were unable to detect increases in CREB or BDNF in response to FLX (Dias *et al.*, 2003). The present study found no difference in the expression of neurotrophins in response to FLX *in vitro*. All of the studies conducted to assess the effect of FLX employed either brain-derived primary cultures or animal models, with generally using much higher concentrations of the drug. These differences may account for the discrepancy between the published data and ours. This may also suggest intrinsic differences between hES-derived NPs and primary cultures of the same type of cells directly isolated from animals or human. Considering the fact that the studies of FLX and tumor cell lines from lymphoma, neuroblastoma, pheochromocytoma and glioblastoma can also increase neurotrophic factors and apoptotic activities (Bartholoma *et al.*, 2002; Hisaoka *et al.*, 2005; Koch *et al.*, 2003; Levkovitz *et al.*, 2005).

Although pharmacotoxicology uses many cell culture systems, current *in vitro* models, such as primary cultures or established cell lines, are quite different from *in vivo* analogs (Gottlieb, 2002; Wobus *et al.*, 1994).

Primary culture of human neural tissue is generally impractical, and while some neuronal cell lines can be induced to express markers of mature neurons under special culture conditions, they often fail to represent the properties of specialized neurons. This is often due, in part, to the origin of these cells in CNS-derived tumors, to a loss of viability, and to changes in phenotypic properties as a result of long-term culture.

Our study can help to elucidate the mechanism of action of FLX as a therapeutic drug for the treatment of depression by providing a valid alternative to current *in vitro* models. Their most attractive characteristic is their capacity to renew indefinitely while maintaining their differentiation potential. It is worth mentioning that standardization may be a challenge when working with hESCs. It has been shown that culture conditions will alter the gene and protein expression in these cells and, as a consequence, variations may occur between replicates of the same study and among studies, even though the cell line might be the same. In addition, the process for generating large numbers of hESCs is labor intensive, and the possibility of irreversible genetic or epigenetic changes during experiments is always present (Pouton and Haynes, 2007). Regardless, hESCs can provide insights into a drug's mechanisms of action and can reveal new targets for currently prescribed pharmaceuticals. Human ESCs may also help predict and avoid potential drug toxicity in humans. It is therefore essential that research and optimization of their use for these purposes continue.

Materials and Methods

All reagents were purchased from Sigma unless otherwise indicated.

Cell culture and differentiation

Human embryonic stem cell culture

Human H9 ESCs (passages 29 to 31) with a stable, normal (46, XX) karyotype were cultured as described previously (Zhang *et al.*, 2001). Briefly, cells were cultured on a mitotically inactive feeder layer of mouse embryonic fibroblasts. The culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM/F12; Invitrogen, Rockville, MD), 20% knockout serum replacement (Invitrogen), 0.1 mM β-mercaptoethanol, 4 ng/ml FGF-2 (Invitrogen), 1% nonessential amino acids (Invitrogen), and 2 mM L-glutamine (Invitrogen). The undifferentiated state of the hESCs was confirmed by characteristic morphology monitoring and immunostaining with anti-Oct4 antibody (Suppl. Fig. 1). All cultures were maintained in a humidified incubator with 5% CO_2 at 37ºC.

Generation of hESC-derived neuronal precursors

A modification of the procedure described by Zhang was used to isolate hESC-derived neuronal precursors (Zhang *et al.*, 2001). Human ESCs colonies were mechanically isolated when they reached a diameter of 1 to 1.5 mm. Intermediate-sized clumps of cells were transferred to 35 mm dishes (BD Biosciences, Bedford, MA), and cultured in ESC medium at 37ºC for two days (Fig. 1A). Cell colonies that grew into nonattached embryoid bodies (EBs) were removed and transferred to new 35 mm dishes.

Floating EBs were cultured in DMEM/F12 with 1% N2 supplement (Invitrogen), medium (basal neural medium) and hESC medium (1:1) for four days. Subsequently, the EBs were transferred into a new dish and

were cultured with 100% basal neural medium plus 3 μM retinoic acid (2+/2-; Fig. 1B and 1C). After ten days, well-demarcated EBs were attached to fibronectin-coated (Invitrogen) plates and cultured for nine days with basal neural medium plus 20 ng/ml FGF-2. A significant number of rosettes developed in the center of the differentiating EBs (Fig. 1D).

Rosette colonies were isolated, using a Pasteur pipette, and dissociated with a micropipette (Fig. 1E). Cells were replated on poly-L-ornithine/laminin-coated plates (50 μg/ml of poly-L-ornithine, 20 μg/ml of laminin) and cultured in basal neural medium containing 10 ng/ml FGF-2 for ten days (Fig. 1F). Subsequently, the cells, now called neuronal precursors (NPs), were cultured as a monolayer in basal neural medium containing 4 ng/ml FGF-2 (Fig. 2). NPs reached proliferative senescence at the tenth passage.

Treatment of hESC-derived neuronal precursors with fluoxetine

Stock solutions of FLX were prepared using DMSO and were diluted directly into the culture media at final FLX concentrations of 0.5 μg/ml. NPs were cultured in DMEM/F12-supplemented N2 culture medium in the presence of FLX. FGF-2 was excluded from the cell culture medium when the cells were treated with FLX. Culture medium was changed every two days and only DMSO was added to controls. NPs were cultured for two weeks in 0.5 μg/ml of FLX, at which point they were evaluated using immunostaining, RT-PCR, western blotting and real-time RT-PCR. For the MTT (3(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) assay, NPs were cultured for various times (for 24, 48, and 72 hrs and for one and two weeks) in different concentrations of FLX (from 0 to 10 μg/ml). Cumulative population doubling level (CPDL) in continual subculture was calculated taking into account the total seeded and the total harvested cells from each culture dish. To determine the CPDL, NPs were cultured for 45 days in 0.5 μg/ml of FLX and passaged at five- to seven-day intervals. For the bromodeoxyuridine (BrdU) incorporation assay, NP cells were cultured for 72 hrs and one week in 0.5 μg/ml FLX. For the staurosporine-(STS-)induced apoptosis assay, NP cells were cultured for 72 hrs, one week, and two weeks in 0.5 μg/ml and 1.0 μg/ml of FLX.

Karyotype analysis

Karyotyping was performed on the NPs using standard methods (G-banding) by Cell Line Genetics (Madison, WI). At least 20 cells from each sample were examined.

Characterization of hESC-derived neuronal precursors

Immunofluorescence and quantification of immunopositive NPs

Protein expression in hESCs and NPs was analyzed by immunofluorescence. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.05% Triton X-100. After blocking with 3% bovine serum albumin, cells were incubated with primary antibodies. The following primary antibodies and dilutions were used: mouse anti-class III β-tubulin-Tuj1, 1:500; rabbit anti-glial fibrillary acidic protein (GFAP), 1:5000; rabbit anti-nestin, 1:1000 (Abcam); mouse anti-cytokeratin-8 (CK-8), 1:200; mouse anti-microtubule-associated protein 2 (MAP2), 1:500; goat anti-Oct4, 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-receptor interacting protein (RIP), 1:1000; mouse anti-polysialylated neuronal cell adhesion molecule (PSA-NCAM), 1:250 (Chemicon, Temecula, CA); and rabbit anti-tyrosine hydroxylase (TH), 1:1000 (Pel-freez, Arkansas). The antigens were visualized under UV light using one of the following secondary antibodies: Alexa Fluor 594 or 488 (Molecular Probes, Eugene, OR). Cells were counterstained with 1 μg/ml of Hoechst 33342 for 10 min at room temperature. Semiquantitative measurement of immunopositive cells was determined using a 20X Zeiss LSC System objective lens mounted on a Nikon TE 2000-U microscope. More than 1,000 immunopositive cells for each primary antibody were photographed and counted. The number and the percentage of immunopositive cells were calculated in relation to the total number of Hoechst-positive cells (>1,000 cells).

RT-PCR

Total RNA was extracted from hESC-derived NPs before and after two weeks with and without FLX, using the Picopure RNA Isolation Kit and following the manufacturer's instructions (Arcturus, Sunnyvale, CA). After DNAse I digestion, cDNA was prepared from 1 μg total RNA, using the SuperScript III RT-PCR Kit (Invitrogen) as instructed by the manufacturer. Primer pair sequences are shown in Suppl. Table 1. The amplification procedure consisted of 30 cycles (denaturation at 94ºC for 30 sec, annealing at 58ºC for 40 sec, and extension at 72ºC for 45 sec). Amplification reactions were conducted in a final volume of 25 μl containing 1.0 μl cDNA, 100 pmol each of forward and reverse primer and of PCR Master Mix (Promega, Madison, WI). RT-PCR products were separated by electrophoresis on 1% agarose gels and stained with ethidium bromide.

Assessment of proliferation, apoptosis, and production of neurotrophic factors in fluoxetine -treated neuronal precursors

MTT assay

MTT assays were performed at 0, 24, 48, and 96 hrs and at one and two weeks after FLX treatment. NPs growing without FLX were used as negative controls. The MTT assays were performed as previously described (Scholz *et al.*, 1999). Briefly, 2 ß 10^4 NPs were seeded on 24-well plates and grown in the presence of FLX at 0, 0.1, 0.5, 1, 10, and 100 μg/ml. MTT (400 μg/ml) was added to each well for a 3 hr incubation period. HCl-isopropanol (0.04 M) was added to the cultures, and cells were incubated again for 15 min at 37ºC. The absorbance at 570 nm was measured using a spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). Results were expressed as the percentage of blue formazan absorbance in treated cells relative to that in cells without FLX. Experiments were run in triplicate.

Cumulative population doubling level (CPDL)

FLX-treated (0.5 μg/ml) NPs were continuously passaged in neural media without FGF2 for 45 days, and each passage was five to seven day interval. The CPDL was calculated to determine their proliferation potential. Nontreated NPs, cultured in same condition but without FLX, were used as controls. The CPDL at each passage was calculated from the cell count using the equation ln(*Nf*/*Ni*)/ln2, where *Ni* and *Nf* are the initial and final cell numbers, respectively, and ln is the natural log(Lin *et al.*, 2005).

5-Bromo-2-deoxyuridine (BrdU) incorporation assay

FLX-treated NP and control cells were cultured overnight with 30 μg/ml of BrdU prior to immunostaining. Cells were fixed with 4% paraformaldehyde and then incubated with blocking buffer (PBS containing 0.05% Triton X-100, 0.5% bovine serum albumin) for 1 hr at room temperature. Following incubation in 2.0 M HCl for 20 min at room temperature, cells were washed with 0.1 M sodium borate buffer. Immunostaining was done using primary mouse anti-BrdU antibody (1:100, Roche Applied Science, Indianapolis, IN), and the secondary antibody was Alexa Fluor 594 anti-mouse antibody (Molecular Probes). The nuclei were counterstained with Hoechst 33342 (1μg/ml) for 10 min at room temperature. The number and the percentage of BrdU-immunopositive cells were calculated in relation to the total number of Hoechst-positive cells (> 1,000 cells).

Staurosporine-induced apoptosis

FLX (0.5 and 1.0 μg/ml) was added to NPs for three days, seven days, and two weeks prior to induction of apoptosis by 200 nM STS. Twenty hrs after induction, cells were fixed with 4% paraformaldehyde and incubated with Hoechst 33342 for 30 min at room temperature. The number of fragmented nuclei with condensed chromatin, which reliably indicates apoptosis, was determined. The percentage of apoptotic cells was

calculated in relation to the total number of Hoechst-positive cells (> 500 cells).

Real time RT- PCR and quantification of transcripts

Isolated total RNA from NPs cultured with and without FLX was spiked with recombinant Venus-GFP (rVenusGFP) RNA (300 fg/μg total RNA) as an exogenous control. One microgram of total RNA was reverse transcribed for 60 min at 42ºC using SuperScript Reverse Transcriptase (Invitrogen) in a 50 μl reaction containing RT buffer (5X) with 500 μM random primers, 500 μM dNTPs, 10 mM DDT and 40 IU RNaseOUT. After heat inactivation of the reverse transcriptase for 15 min at 70ºC, samples were diluted to a concentration of 10 ng/μl of input RNA, using nuclease-free water. Quantities of transcripts were detected by real-time RT-PCR, using an ABI 7000 Sequence Detection System and SYBR green chemistry (Applied Biosystems, Foster, CA). The primer pairs were designed using Primer Express Software (Applied Biosystems) and human sequences available on the GenBank database (Suppl. Table 2). After optimizing the reaction conditions for'all primer pairs, 25 μl PCR reactions were run in triplicate for each sample. Each reaction mixture consisted of SYBR Green PCR Master Mix (Applied Biosystems), 600 nM of sequence-specific primer pairs, 10 ng of input RNA equivalent template cDNA and water. For each primer pair, no-RT and no-template samples were run as controls. The thermal cycling profile of the reactions included one cycle at 60ºC for 2 min, one cycle at 95ºC for 10 min, and 40 cycles at 95ºC for 15 sec and at 60ºC for 1 min. Melting curve analyses were run at the end of each PCR reaction to monitor the PCR product sizes. The relative difference in the initial amount of each cDNA sample was determined by comparing the C_T values. To quantify the mRNA concentrations, standard curves for each gene were generated using five 1/10 serial dilutions from a single stock of human genomic DNA and human reference cDNA mixture (10 pg to 100 ng). Before the statistical analyses, the amount of each transcript was normalized to the amount of exogenous control transcript (rVenusGFP) in the same sample.

Western blotting

NP pellets from cells incubated with and without FLX were collected and kept at 80ºC until used for western blotting. Briefly, cell lysates were prepared in TNE lysis buffer (50 mM Tris, 2 mM EDTA, 5 mM $Na_2P_2O_7$, 100 μM Na_3VO_4, 5 mM NaF, 150 mM NaCl, 1% NP-40, 1μg/ml aprotinin, 1 μg/ml leupeptin and 174 μg/ml PMSF), incubated on ice for 30 min, and centrifuged at 13,000 rpm for 30 min. Supernatants were evaluated to measure the amount of total protein using the Quick Start Bradford Protein Assay Kit (Bio-Rad, Inc., Hercules, CA). Cell lysates were then treated with SDS sample buffer(Sambrook and Russell, 2001). We loaded approximately 13 μg of total protein into each lane of either 10% or 12% SDS-polyacrylamide gels. Proteins were separated by electrophoresis at 50 V for 4 to 6 hrs in a tris-glycine buffer and transferred onto PVDF membrane at 4ºC, 100 mA for 6 hrs in a transfer buffer. Blots were blocked in 5% skim milk in tris-buffered saline and 0.1% Tween 20 (TBST) with agitation for 90 min at room temperature. Primary antibodies were diluted in TBST with 3% skim milk, using the manufacturer's recommended concentrations: mouse anti-CREB (1:1000, Chemicon), mouse anti-pCREB (Ser133) (1:1000, Cell Signaling Technology, Danvers, MA), mouse anti-5HTT (1:500, Advance Targeting Systems, San Diego, CA), mouse anti-BDNF (1:500; Abcam), and mouse anti-β actin (1:1000; Sigma). Blots were incubated overnight at 4ºC with each primary antibody and subsequently washed with TBST. Alkaline phosphatase-conjugated goat anti-mouse antibody (Bio-Rad) was diluted 1:3000 in TBST with 3% skim milk. The blots were incubated with the secondary antibody at room temperature for 90 min, and then extensively washed with TBST. Blots were immersed in NBT/BCIP substrate (Roche Applied Science) until the results were visualized. The images were generated by scanning and were analyzed using a densitometer (Bio-Rad).

Statistical analysis

One-way analysis of variance (ANOVA) and least significant difference (LSD) procedures were used for the statistical analyses. Quantitative RT-PCR results were analyzed using expression values normalized against human *GAPDH*. The remaining data analysis employed two-tailed Student's *t*-test. Each experiment was conducted at least three times ($n \geq 3$) and a p value of less than 0.05 ($p \leq 0.05$) was considered significantly different.

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