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Fluoxetine-induced proliferation and differentiation of neural progenitor cells isolated from rat postnatal cerebellum

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ABSTRACT

Previous studies have shown that the serotonin-reuptake inhibitor (SSRI) fluoxetine affects neural progenitors derived from postnatal cerebellum or adult hippocampus and stimulates their proliferation. In the human cerebellum, the proliferation of cerebellar granule cells (CGC) continues until the 11th postnatal month and could be influenced in infants by breastfeeding-delivered SSRIs. Current information about fluoxetine effects on postnatal cerebellar neural progenitors is limited. Here we report the characterization of fluoxetine actions on rat postnatal cerebellar neural progenitors. RT-PCR and immunostaining revealed the expression of serotonin transporter (SERT), $5HT_{1A}$ receptors, tryptophan hydroxylase (TPH), and serotonin (5HT). Protracted in vitro fluoxetine treatment increased cell proliferation and differentiation. The proliferative effects of fluoxetine, 5HT, and the selective agonist of $5HT_{1A}$ receptors *trans*-8-hydroxy-2-(*N*-*n*-propyl-*N*-3′-iodo-2′-propenyl)aminotetralin (8-OH-PIPAT) were abolished by the selective antagonist of $5HT_{1A}$ receptors, *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride (WAY-100635). Furthermore, fluoxetine-induced activation of both the cAMP-response element-binding (CREB) protein and extracellular signal-regulated protein kinases (ERK1/2), which was abolished by the selective inhibitor of MAP kinase kinase (MEK) 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126), and increased cyclin D1 expression. All these effects were prevented by WAY-100635. Collectively, our results demonstrate that rat postnatal cerebellum contains neural progenitors capable of proliferating and differentiating in response to fluoxetine exposure, possibly through the activation of $5HT_{1A}$ receptors. The relevance of these findings for possible SSRI effects on the developing postnatal/infant human cerebellum should be explored.

1. Introduction

The cerebellum is one of the brain regions exhibiting protracted postnatal neurogenesis. Cerebellar granule cells (CGC) are generated almost entirely postnatally from granule cell progenitors located subpially in the external granule layer. This transitory germinal layer is active up to postnatal day 21 in mice, 22 in rats, and up to postnatal month 3 in primates

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and 11 in humans (for review, see [1]). Recently, it has been shown that stem cells can be isolated from both postnatal [2] and adult [3,4] cerebellum, suggesting that latent adult neurogenic potential may be present in this brain structure. Thus, it is possible that quiescent cerebellar neural progenitors could be mobilized, e.g., pharmacologically, for brain repair and other therapeutic purposes. Hence, it was shown that in primary CGC cultures prepared from a nearly week-old rat cerebellum, protracted treatment with well-known antidepressant compounds, e.g., selective serotonin-reuptake inhibitors (SSRIs) including fluoxetine, significantly increased cell proliferation and differentiation [5–7]. Although no data are available on whether fluoxetine affects quiescent cerebellar neural progenitors in the adult brain, the effects of this drug on early postnatal neural progenitors may be clinically relevant. Namely, these progenitors are active in the human cerebellum up to 11 months postnatally [8] and fluoxetine and its active metabolite norfluoxetine can accumulate in nursing infants via breastfeeding (for review, see [9]). There is growing evidence that SSRI exposure during pregnancy and lactation may induce deficits in postnatal motor development and motor control, both in humans [10] and rodents [11,12]. Furthermore, SSRIs including fluoxetine are widely used as a treatment for infantile autism [13–15].

Additionally, in cultured neural stem cells (NSC) derived from the hippocampus of adult rats, in vitro fluoxetine treatment increased the NSC proliferation rate [16]. Hence, it appears that antidepressants may exert a general neurogenic effect on cell precursors/NSC in various brain regions. This effect suggests the concept that a crucial component of the antidepressant action of drugs that increase serotonergic neurotransmission in the adult brain, is their capacity to upregulate cell proliferation and neurogenesis in the dentate gyrus of the hippocampus [5,17–21]. Furthermore, it was shown that increased adult hippocampal de novo neuronal proliferation may be critically involved in antidepressant activity and that the $5HT_{1A}$ receptor activation is required during fluoxetine-induced cell proliferation, survival, and differentiation [22]. However, the participation of neurogenesis and $5HT_{1A}$ receptors in antidepressant action may not be operative in all experimental models and/or species [23].

The specific intracellular signal transduction cascades that influence antidepressant-triggered neurogenesis are still largely unknown. It has been suggested that upregulation of the transcription factor cAMP-response element-binding (CREB) protein and the brain-derived neurotrophic factor (BDNF) could be involved [20,24]. Most antidepressants that increase neurogenesis also modulate the expression, phosphorylation, and transcriptional activity of CREB [25,26], which at least partly mediates the downstream induction of BDNF gene expression both in vitro and in vivo [24,25,27]. Furthermore, neuronal CREB function is influenced by the extracellular signal-regulated protein kinase (ERK1/2)/mitogen-activated protein kinase (MAPK) kinase pathway. The sustained phosphorylation profile of CREB, needed to induce changes in the expression of endogenous CRE-driven genes, is maintained through activation of the ERK/MAPK cascades [28,29]. Moreover, the ERK/MAPK signaling pathway plays a crucial role in cell proliferation via the upregulation of the expression of cyclin D1 [30].

Our previous work has provided the first indication that in early postnatal rat CGC cultures, SSRIs may directly affect the proliferation of a population of neural progenitors negative to either neuron or glial markers [7]. Here we further investigated the effects of fluoxetine on these cells.

2. Materials and methods

2.1. Materials

Serotonin hydrochloride, fluoxetine hydrochloride, and *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride (WAY-100635) were purchased from Sigma (St. Louis, MO). *trans*-8-hydroxy-2-(*N*-*n*-propyl-*N*-3′-iodo-2′-propenyl)aminotetralin (8-OH-PIPAT) and 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) were purchased from Tocris (Ellisville, MO) and Promega (Madison, WI), respectively. U0126 was dissolved in dimethyl sulfoxide (DMSO; 0.2% final concentration in culture medium). All the other drugs were freshly dissolved in phosphate buffered saline (PBS). All cell culture products were from Sigma, unless otherwise specified. All antibodies were from Chemicon International Inc. (Temecula, CA), unless specified otherwise. All PCR amplification primers were obtained from Sigma-Genosys (Cambridge, UK). All other reagents and solvents were of analytical grade.

2.2. Isolation of neural progenitors from primary cultures of rat cerebellar granule cells (CGC)

For isolation and expansion of the progenitor component, CGC were prepared from the cerebella of 7-day-old rat pups (Sprague–Dawley) as previously described [7]. The cells were plated in 2% gelatin-coated dishes and cultured in serum-free neurobasal medium containing B27 supplement (Gibco, Life Technologies, Rockville, MD), supplemented with 20 ng/ml basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (*growth medium*). In these conditions, glial cells stuck to the gelatin substrate, while neurons remained in suspension and died in a few days. After 10 days in vitro (DIV), neural progenitors lifted off the substrate and floated in suspension as small aggregates (neurospheres), which were harvested and mechanically dissociated to produce a single-cell suspension for replating. Animal handling was performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and with the Italian legislation on animal experimentation (Decreto Legislativo 116/92).

2.3. [^{3}H]thymidine assay of cell proliferation

To determine drug effects on cell proliferation, isolated cerebellar neural progenitors were plated into 24-well culture plates in *growth medium*. They were cultured in the absence or presence of drugs for 72 h and exposed to 1 μCi/ml [^{3}H]thymidine (specific activity 25 Ci/mmol; Amersham Biosciences, Piscataway, NJ) for the last 6 h of the incubation period. Cell-incorporated radioactivity was measured by liquid scintillation counting as previously described [5,7].

2.4. Differentiation conditions

For the differentiation assay, the isolated cerebellar neural progenitors at 10 DIV were plated onto poly-D-lysine-coated glass coverslips in Eagle's basal medium, lacking growth factors and containing 10% fetal calf serum (Celbio, Milano, Italy), 2 mM L-glutamine, 50 μg/ml gentamicin, and 25 mM KCl (*differentiation medium*). Three and 5 days after plating in *differentiation medium* in the absence and presence of 1 μM fluoxetine, cultures were processed for immunocytochemical detection of neuronal, glial, and oligodendroglial antigens as detailed below (Section 2.5).

2.5. Immunocytochemistry

For the assay of cell immunoreactivity for markers typical of either immature or mature neural lineages and for the differentiation assay, the immunocytochemistry was performed in isolated cerebellar neural progenitors at 10 DIV, plated onto 2% gelatin-coated glass coverslips and cultured for 18 h in *growth medium* or for 3 and 5 days in *differentiation medium*, respectively. Cells were then fixed with 4% paraformaldehyde in PBS, pH 7.4, for 10 min at room temperature. After extensive rinsing with PBS, the cells were blocked in blocking buffer (3% bovine serum albumin and 0.1% Triton X-100 in PBS) for 15 min, incubated overnight at 4 °C with primary antibodies diluted in blocking buffer, washed 3 times in PBS, incubated with a secondary antibody diluted in PBS for 1 h at room temperature, washed 3 times in PBS, and mounted in Mowiol mounting medium. The following primary antibodies (dilution in brackets) were used: mouse anti-nestin (1:500); mouse anti-polysialylated neural cell adhesion molecule (PSA-NCAM; 1:500; a generous gift of Seki T.); mouse anti-βIII-tubulin (1:500); mouse anti-neurofilament (NF; 1:1000); rabbit anti-glial fibrillary acidic protein (GFAP; 1:1000); mouse anti-O4 (1:200; Upstate Biotechnology Inc., Lake Placid, NY); rabbit anti-SERT (1:50; Santa Cruz Biotechnology Inc.); rabbit anti-$5HT_{1A}$ (1:10; Santa Cruz Biotechnology Inc.); mouse anti-tryptophan hydroxylase (TPH; 1:750; Sigma); and rabbit anti-serotonin (5HT; 1:1000). AlexaFluor594-coniugated goat anti-mouse and anti-rabbit (1:1000; Molecular Probes, Rockville, MD), and AlexaFluor 488-coniugated goat anti-mouse secondary antibodies (1:1000; Molecular Probes) were used. For quantitative analysis, a nuclear 4,6-diamidino-2-phenylindole (DAPI) counter-stain was used to visualize all cells. For this assay, the coverslips were incubated in 0.5 μg/ml DAPI in PBS for 3 min at 37 °C after the final wash and then washed 3 additional times in PBS. Nine coverslips per control and treated group were considered and five fields for each well (the four quadrants and the center) were imaged by using a digital camera (DC 200) connected to a fluorescence microscope (DMR), both from Leica Imaging Systems (Cambridge, UK). Cell counts were performed with the QWin Image Analysis Software (Leica Imaging Systems). The relative numbers of cells expressing either the immature neuronal antigens nestin, PSA-NCAM, and βIII-tubulin or expressing SERT, $5HT_{1A}$, TPH, and 5HT were expressed as percentages of the total DAPI-stained cell population. For quantitative analysis of cell differentiation in control and fluoxetine-treated cells, the relative numbers of cells expressing different antigens typical of neurons (i.e. NF-positive cells, NF^+), astrocytes (i.e. GFAP-positive cells, $GFAP^+$), and oligodendrocytes (i.e. O4-positive cells, $O4^+$) were expressed as percentages of the total DAPI-stained cell population. The extent of immunoreactivity for SERT, $5HT_{1A}$, TPH, and 5HT in fluoxetine-treated cells was evaluated by fluorescence density of 5 cells in each field (analyzed with ImageJ software, National Institutes of Health, Bethesda, MD) and normalized to the fluorescence intensity of control cells.

2.6. Western blot analysis

For the preparation of total cell extracts, isolated cerebellar neural progenitor cells at 10 DIV were washed with PBS and solubilized in ice-cold lysis buffer (10 mM Tris–HCl, pH 7.4, 75 mM NaCl, 10 mM EDTA, 0.75% deoxycholate, 0.5% sodium dodecyl sulfate, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1.25 mM NaF, and 1 mM sodium pyrophosphate). Cell lysates were centrifuged at 13 000 × *g* for 15 min at 4 °C to remove debris. Protein concentration was measured according to Lowry et al. [31]. Approximately 20 μg of proteins were loaded onto each lane, separated on a 10% sodium dodecyl sulfate-polyacrilamide gel, and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences) at 200 mA for 2 h. Membranes were blocked for 1 h at room temperature in a blocking solution containing 5% non-fat dry milk, 0.1% Tween-20, and Tris-buffered saline (50 mM Tris–HCl, pH 7.5, 150 mM NaCl). After blocking, membranes were incubated overnight at 4 °C with the following primary antibodies (dilution in brackets): anti-CREB polyclonal antibody (1:500; Upstate Biotechnology Inc.); anti-phosphorylated CREB (pCREB) polyclonal antibody specific for Ser^{133} (1:500; Upstate Biotechnology Inc.); anti-ERK1 polyclonal antibody (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA); anti-phosphorylated ERK1/2 (pERK1/2) monoclonal antibody (1:1000; Santa Cruz Biotechnology Inc.); anti-BDNF polyclonal antibody (1:1000); anti-cyclin D1 polyclonal antibody (1:100; Sigma); and anti-β-actin monoclonal antibody (1:1000; Sigma), diluted in blocking solution. After three washes for 10 min each with 0.1% Tween-20 in Tris-buffered saline, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (1:1000; Dako Cytomation, Glostrup, Denmark) for 1 h at room temperature. Following washes (3 min × 10 min) with Tris-buffered saline, the resulting antigen–antibody–peroxidase complexes were detected by enhanced chemiluminescent autoradiography (ECL kit, Amersham Biosciences) and visualized by exposure to Hyperfilm ECL (Amersham Biosciences). ImageJ software (National Institutes of Health, Bethesda, MD) was used to quantify the integrated densities of each band on film.

2.7. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) conditions

Total RNA was extracted from cell pellets (1.5×10^6 cells) by the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions. RNA

integrity and quantity were determined by an RNA 6000 Nano assay in an Agilent BioAnalyser (Agilent Technologies Inc., Santa Clara, CA). RT was performed with Superscript III reverse transcriptase with 500 ng of total RNA and random oligonucleotides (Invitrogen, Carlsbad, CA). PCR was performed in a MX 3000P thermal cycler (Stratagene) in a final volume of 30 μl, with HotStar Taq Polymerase (Quiagen, Hilden, Germany). Amplicons were produced using the following primer pairs: β-actin 5′-TGA ACC CTA AGG CCA ACC GTG-3′ forward (F) and 5′-CTC ATA GCT CTT CTC CAG GG-3′ reverse (R) (395 bp); SERT 5′-TAT GGA ATC ACT CAG TTC TGC-3′ F and 5′-TAA TGC GCT CCT TAA GTG TCC-3′ R (280 bp); TPH 5′-GCA GAG CTG GCT ATG AAC TAC-3′ F and 5′-CTT GGG ATC AAA GGG CTT AAC-3′ R (627 bp); $5HT_{1A}$ 5′-TGC TCA TGC TGG TTC TCT ACG-3′ F and 5′-AGC CTA GCC AGT TAA TTA TGG-3′ R (543 bp); $5HT_{1B}$ 5′-CTG CTA AAA GAA CTC CCA AAA-3′ F and 5′-TTG GGT GTC TGT TTC AAA ATC-3′ R (271 bp); $5HT_{1D}$ 5′-TGT CTC TGG TTT TAA AAG CTC-3′ F and 5′-AGT GTT TCT CTC TTC CCA CA-3′ R (453 bp); $5HT_{2A}$ 5′-TCG AAC TGG ACA ATT GAT GC-3′ F and 5′-GAT GAC AGA GAA CTC TGA GG-3′ R (743 bp); $5HT_{2C}$ 5′-CGT AAT CCT ATT GAG CAT AGC C-3′ F and 5′-CGA ACA GGA GGC TTT TTG TC-3′ R (722 bp). PCR products were analyzed by electrophoresis on 1% agarose gel and data were analyzed in a Molecular Imager VersaDoc System (Bio-rad Laboratories Inc., Hercules, CA).

2.8. *Real-time RT-PCR conditions*

The real-time RT-PCR reaction was performed in a MX 3000P thermal cycler in a final volume of 25 μl containing 100 nM of each primer and 1× Brilliant SYBR Green QPCR Master Mix (Stratagene). The PCR cycling conditions were 10 min of denaturation at 95 °C, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, followed by a dissociation thermal profile of 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. The following primer pairs were used for: BDNF, 5′-CTG GAT GAG GAC CAG AAG GTT C-3′ F and 5′-CAT CAC CCG GGA AGT GTA CAA-3′ R (75 bp); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CAA GGT CAT CCA TGA CAA CTT TG-3′ F and 5′-GGG CCA TCC ACA GTC TTC TG-3′ R

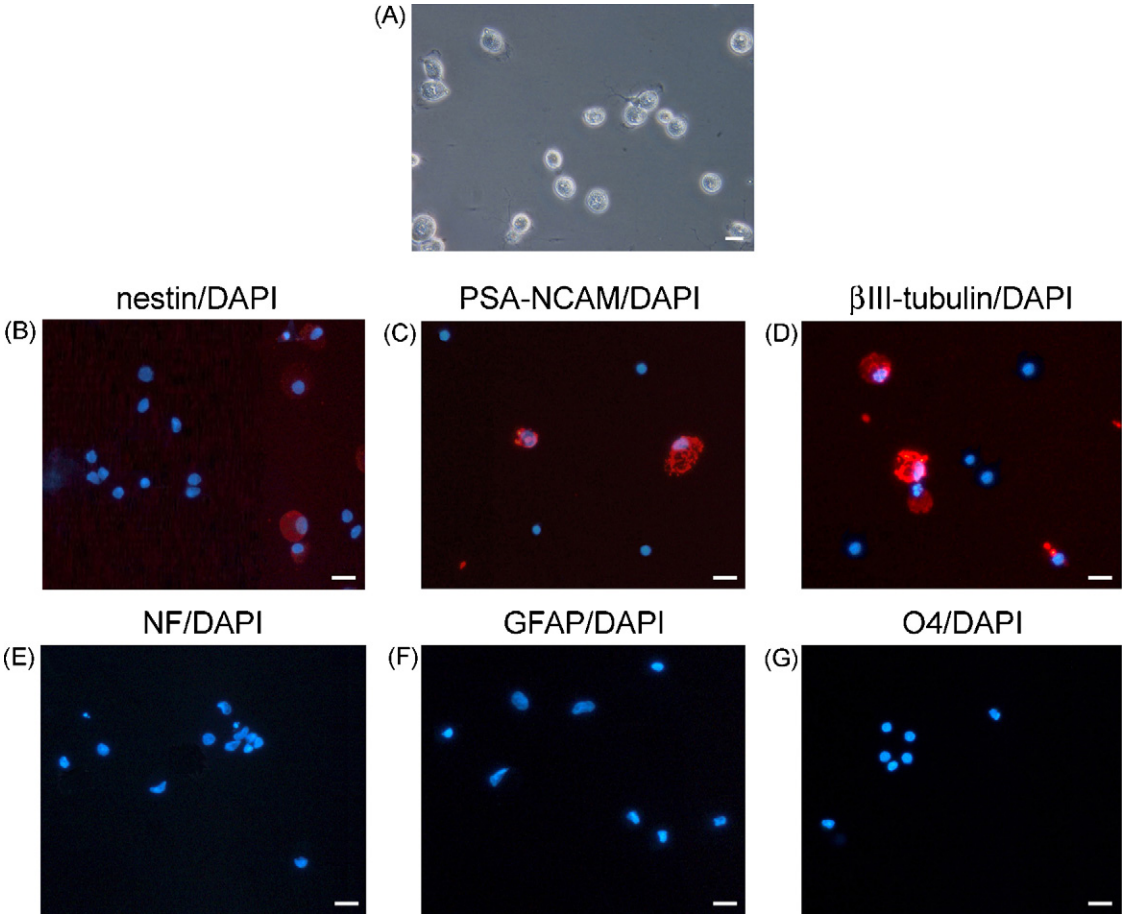

Fig. 1 – Morphology and undifferentiated state of isolated cerebellar neural progenitors. Panel (A) shows the phase-contrast microphotograph taken from a typical dish of cerebellar neural progenitors maintained in *growth medium* for 10 DIV. Panels (B–D) show representative fluorescent microphotographs of the specific immunoreactivity (shown in red) of neural progenitors (maintained as in panel (A)) for nestin (B), PSA-NCAM (C), and βIII-tubulin (D). Panels (E–G) show representative fluorescent microphotographs of the negative immunoreactivity (absence of red) of neural progenitors (maintained as in panel (A)) for NF (E), GFAP (F), and O4 (G). Blue DAPI staining shows the nuclei. Scale bars = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(89 bp); cyclin D1, 5′-AAC ATG CAC AGA CCT TTG TGG CC-3′ F and 5′-ACA CTC CCA GCA GCC ACC AT-3′ R (85 bp). Amounts of each gene product were calculated using linear regression analysis from standard curves, demonstrating amplification efficiencies ranging from 90 to 100%. Dissociation curves were generated for each primer pair showing single product amplification.

2.9. Statistics

All data are expressed as the mean ± S.D. Groups were compared by ANOVA, followed by post hoc tests for either selected or multiple comparisons; $p < 0.05$ was taken as significant.

3. Results

3.1. Immunocytochemical characterization of isolated cerebellar neural progenitors

Isolated cerebellar neural progenitor cells, when maintained in *growth medium*, displayed a round shape (Fig. 1A) and an undifferentiated state evidenced by their immunoreactivity for several immature markers of neuronal lineage. A minor percentage (18 ± 4.2%) of cells were immunoreactive for nestin (Fig. 1B), a protein characteristic of undifferentiated neuroepithelial cells [32] and 30 ± 2.5% of the cells showed immunoreactivity for PSA-NCAM (Fig. 1C), a cell-surface antigen of neuron-restricted progenitors, which is expressed by migrating neuroblasts [33]. A higher percentage of the cells (52 ± 3.6%) were immunoreactive for βIII-tubulin (Fig. 1D), a specific marker for developing neurons [34]. None of the cells studied exhibited immunoreactivity to markers typical of mature neurons (NF, Fig. 1E), astrocytes (GFAP, Fig. 1F) or oligodendrocytes (O4, Fig. 1G).

3.2. Effects of fluoxetine and 5HT receptor agonists and antagonists on the proliferation of cerebellar neural progenitors

The analysis of the concentration dependency of the effects on cell proliferation induced by protracted (72 h) treatment of isolated cerebellar neural progenitors at 10 DIV with increasing concentrations (0.001–20 μM) of fluoxetine, 5HT, the selective agonist of $5HT_{1A}$ receptors 8-OH-PIPAT [35], and the selective antagonist of $5HT_{1A}$ receptors WAY-100635 is shown in Fig. 2. Both fluoxetine and 5HT significantly increased cell proliferation only at the concentration of 1 μM, whereas 8-OH-PIPAT produced a maximum increase in cell proliferation at 100 nM. At the highest concentration tested (20 μM), all compounds except WAY-100635 significantly decreased cell proliferation. WAY-100635 was ineffective at any of the concentrations tested.

The proliferative response induced by the 72-h exposure of cerebellar neural progenitor cells at 10 DIV to either 1 μM fluoxetine, 1 μM 5HT, or 100 nM 8-OH-PIPAT was abolished by 1 nM WAY-100635 (Fig. 3), a selective antagonist of $5HT_{1A}$ receptors when used at nanomolar concentrations [36].

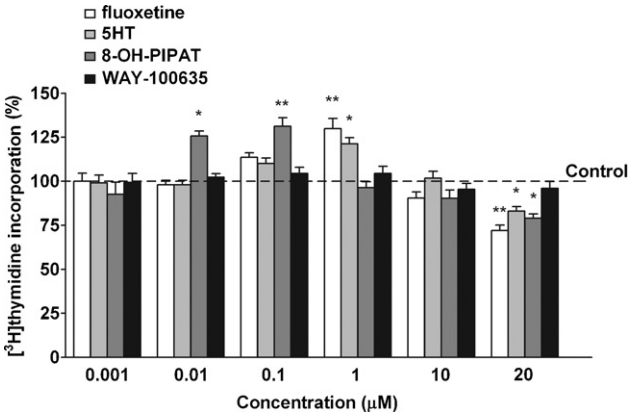

Fig. 2 – Effects of increasing concentrations of fluoxetine, 5HT, 8-OH-PIPAT, and WAY-100635 on the proliferation of isolated cerebellar neural progenitors. Cerebellar neural progenitors were isolated and plated into gelatin-coated dishes containing the *growth medium*. After 10 DIV, cells were incubated for 72 h with increasing concentrations (0.001–20 μM) of fluoxetine, serotonin (5HT), 8-OH-PIPAT, or WAY-100635. Cell proliferation was assayed as [^{3}H]thymidine incorporation. Data are expressed as a percentage of vehicle-treated controls and are the mean ± S.D. of at least 4 determinations from 5 independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control (ANOVA followed by the Dunnett's multiple comparison test).

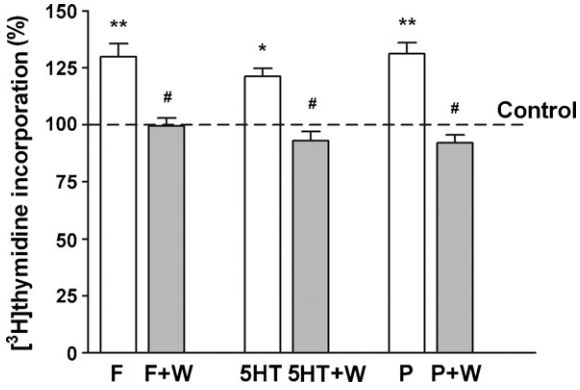

Fig. 3 – The serotonin receptor antagonist WAY-100635 prevents the stimulatory action of protracted fluoxetine, 5HT, and 8-OH-PIPAT treatment on the proliferation of isolated cerebellar neural progenitors. Cerebellar neural progenitors were isolated and plated into gelatin-coated dishes containing the *growth medium*. After 10 DIV, cells were treated for 72 h with 1 μM fluoxetine (F), 1 μM serotonin (5HT), or 100 nM 8-OH-PIPAT (P) in the absence or presence of 1 nM WAY-100635 (W). Cell proliferation was assayed as [^{3}H]thymidine incorporation. Data are expressed as a percentage of vehicle-treated controls and are the mean ± S.D. of at least 4 determinations from 5 independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control and $^{\#}p < 0.05$ vs. the corresponding values obtained from cells treated in the absence of WAY-100635 (ANOVA followed by the Dunnett's multiple comparison test).

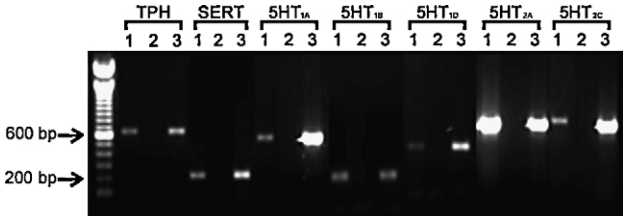

Fig. 4 – The presence of mRNAs for serotonergic markers in cerebellar neural progenitors. Samples were collected from cerebellar neural progenitors after 10 DIV. The representative gel electrophoresis results of the PCR products are shown, demonstrating the presence of tryptophan hydroxylase (TPH), serotonin transporter (SERT), and serotonin (5HT) receptor subtypes. Lane 1, isolated cerebellar neural progenitors; lane 2, negative control (water); lane 3, positive control (rat total brain cDNA).

3.3. *Presence of mRNAs and expression of serotonergic markers in cerebellar neural progenitors*

To clarify the involvement of the serotonergic system in the neural progenitors' ability to respond to fluoxetine, 5HT, and 8-OH-PIPAT treatment in a $5HT_{1A}$ antagonist-sensitive manner, we investigated whether these cells are capable of producing 5HT and whether they express 5HT receptors (in particular, $5HT_{1A}$ receptors) and serotonin transporter (SERT). We found evidence for the presence of mRNAs for TPH (the rate-limiting enzyme in 5HT synthesis), SERT, and various subtypes of 5HT receptors, including $5HT_{1A}$, $5HT_{1B}$, $5HT_{1D}$, $5HT_{2A}$, and $5HT_{2C}$ (Fig. 4). Furthermore, staining with specific antibodies confirmed that $67 \pm 3.6\%$ of these cells contained either SERT (Fig. 5A) or $5HT_{1A}$ receptors (Fig. 5B). In addition, $80 \pm 2.3\%$ of the cells contained either TPH protein (Fig. 5C) or 5HT (Fig. 5D). Fluoxetine treatment did not cause any significant differences in immunoreactivity for SERT, $5HT_{1A}$ receptors, and TPH compared to the corresponding vehicle-treated control (data not shown). However, fluoxetine treatment caused a 40% decrease in the intensity of 5HT-immunoreactivity (evaluated as fluorescence density, as detailed in Section 2) compared to the control. At the same time, the percentage of cells immunopositive for 5HT (Fig. 5E) was not affected by fluoxetine treatment.

3.4. *Fluoxetine-induced increases in pCREB, pERK1/2, and cyclin D1 mRNA and protein levels in cerebellar neural progenitors*

Under growth conditions, protracted fluoxetine treatment, which stimulates cell proliferation (Fig. 3), increased the phosphorylation state of CREB and ERK1/2 proteins (Fig. 6), suggesting the activation of the ERK/MAPK mitogenic pathway. Thus, at 10 DIV, progenitor cells were treated with 1 μM fluoxetine for increasing periods of time (6, 24, and 48 h), in the absence or presence of 1 nM WAY-100635. Fluoxetine treatment had no significant effect on total content of either CREB (Fig. 6A) or ERK1/2 (Fig. 6C) proteins. Whereas 6- and 24-h fluoxetine treatment did not change the phosphorylation states of CREB and ERK1/2, 48-h treatment induced a significant increase in both pCREB (Fig. 6A and B) and pERK1/2 (Fig. 6C and D) levels. These stimulatory effects of fluoxetine were completely counteracted by WAY-100635.

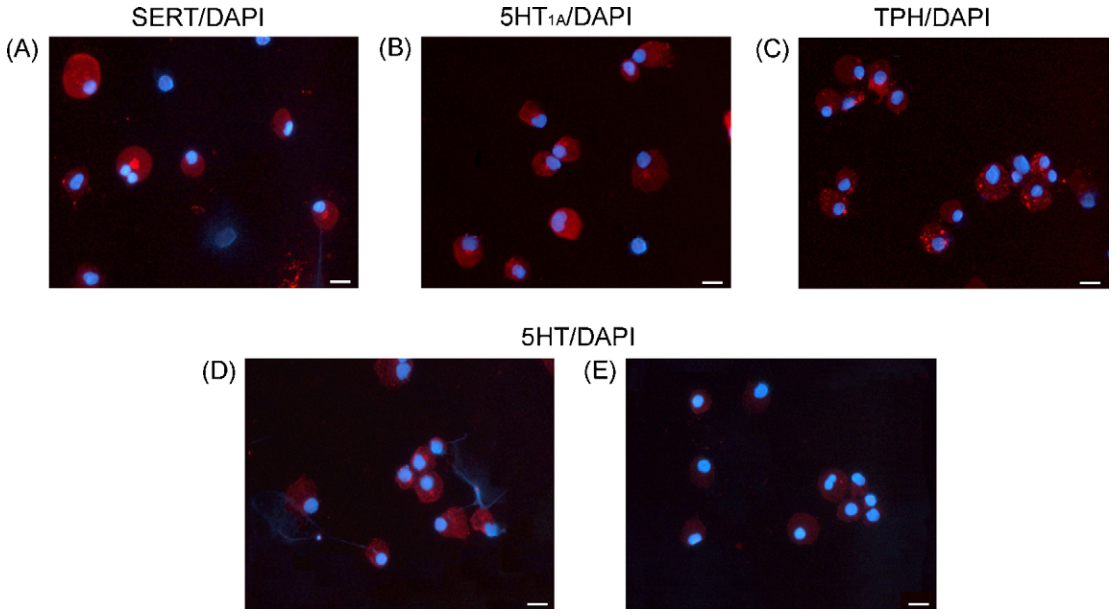

Fig. 5 – The expression of serotonergic markers in cerebellar neural progenitors. Panels (A–D) show representative fluorescent microphotographs of the immunoreactivity of neural progenitors maintained in *growth medium* for 10 DIV for serotonin transporter (SERT; A), $5HT_{1A}$ receptor (B), tryptophan hydroxylase (TPH; C), and serotonin (5HT; D). Panel (E) shows a representative fluorescent microphotograph of the immunoreactivity of fluoxetine-treated neural progenitors for 5HT. Blue DAPI staining shows the nuclei. Scale bars = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

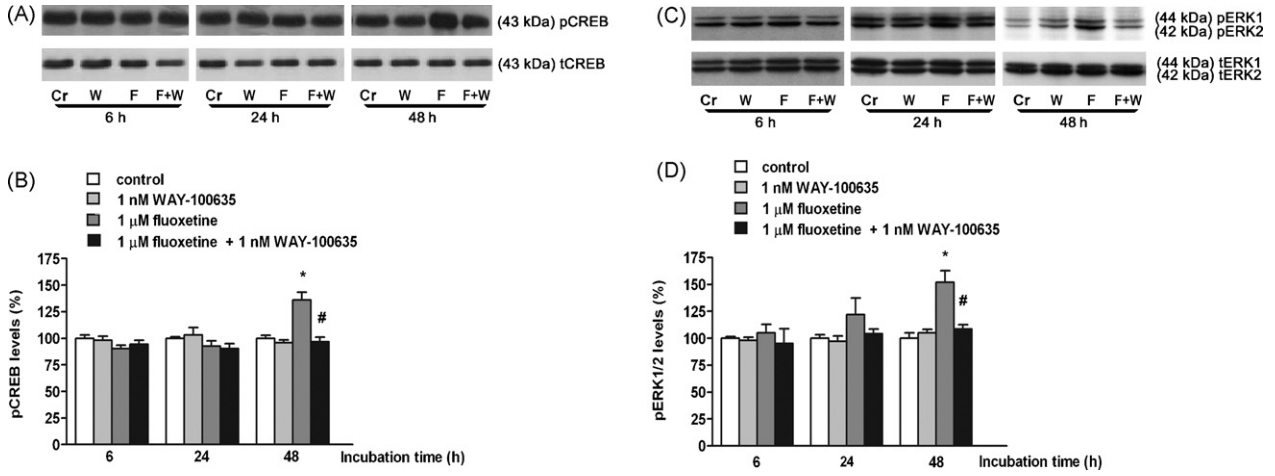

Fig. 6 – WAY-100635 prevents the stimulatory action of a protracted fluoxetine treatment on pCREB and pERK1/2 levels. At 10 DIV, cerebellar neural progenitors maintained in *growth medium* were exposed for 6, 24, and 48 h to 1 μM fluoxetine (F) in the absence or presence of 1 nM WAY-100635 (W). The corresponding vehicle-treated cultures were used as controls (Cr). The levels of total and phosphorylated proteins were measured by Western immunoblotting. Representative immunoblots of phosphorylated (pCREB) and total (tCREB) CREB are shown in (A), and immunoblots of phosphorylated (pERK1/2) and total (tERK1/2) ERK are shown in (C). Bar graphs depict the results of a densitometric quantification from immunoblots of amounts of pCREB (B) and pERK1/2 (D), normalized to the corresponding total protein. The results (mean ± S.D. of at least 4 determinations from 3 independent experiments) are expressed as percentage of their corresponding controls. $^{*}p < 0.05$ vs. corresponding control and $^{\#}p < 0.05$ vs. 1 μM fluoxetine (ANOVA and Bonferroni's test for selected comparisons).

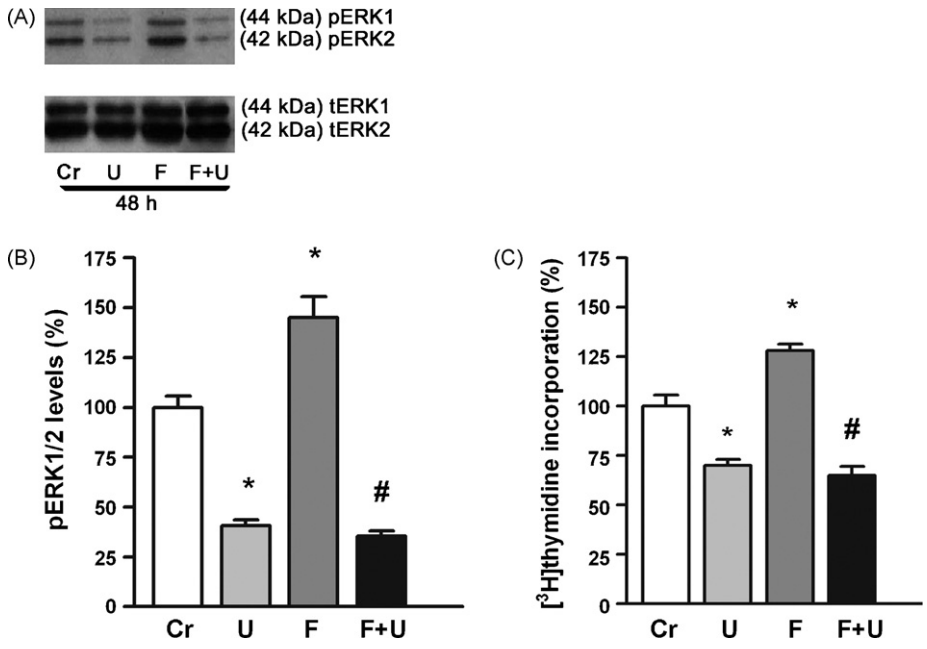

Fig. 7 – U0126 prevents the increase in pERK1/2 and proliferation of isolated cerebellar neural progenitors induced by a 48-h fluoxetine treatment. Cerebellar neural progenitors maintained in *growth medium* for 10 DIV were pretreated with vehicle (Cr) or 10 μM U0126 (U) for 1 h before a 48-h incubation in the absence or presence of 1 μM fluoxetine (F). Representative immunoblots of phosphorylated (pERK1/2) and total (tERK1/2) ERK are shown in (A). Bar graphs depict the results of a densitometric quantification from immunoblots of amounts of pERK1/2 (B), normalized to the corresponding total protein. Cell proliferation (C) was assayed as [^{3}H]thymidine incorporation. Data are expressed as a percentage of vehicle-treated controls and are the mean ± S.D. of at least 4 determinations from 5 independent experiments. $^{*}p < 0.05$ vs. control and $^{\#}p < 0.05$ vs. 1 μM fluoxetine (ANOVA followed by the Dunnett's multiple comparison test).

Treatment with WAY-100635 alone did not affect either total content or the phosphorylation states of CREB and ERK1/2 proteins at any time point tested (Fig. 6).

The increases in pERK1/2 and cell proliferation induced by 48-h fluoxetine treatment were completely prevented by 1-h pretreatment with 10 μM U0126, a selective inhibitor of MAP kinase kinase (MEK) [37] (Fig. 7). Furthermore, treatment with U0126 alone significantly reduced the basal levels of pERK1/2 (Fig. 7A and B) and cell proliferation (Fig. 7C).

Prompted by reports showing that antidepressant-induced activation of CREB may lead to downstream induction of BDNF gene expression [24,25,27] and that the ERK/MAPK signaling pathway plays a crucial role in cell proliferation via upregulating the expression of cyclin D1 [30], we assayed the mRNA levels and expression of both BDNF and cyclin D1 proteins. Fluoxetine treatment (up to 72 h) of cerebellar neural progenitors maintained under growth conditions did not significantly change either BDNF mRNA or protein levels (data not shown). On the other hand, a 48-h treatment with fluoxetine induced a significant increase both in cyclin D1 mRNA (Fig. 8A) and protein (Fig. 8B and C) levels. The fluoxetine-induced increase in cyclin D1 mRNA and protein levels was prevented by WAY-100635 (Fig. 8). WAY-100635 alone did not affect cyclin D1 mRNA and protein levels (Fig. 8).

3.5. *Multipotency of cerebellar neural progenitors*

Isolated cerebellar neural progenitor cells, when replated and maintained under differentiation conditions for 3 and 5 days, spontaneously gave rise to three phenotypes, astrocytes, oligodendrocytes, and neurons, as revealed by immunocytochemical analysis (Fig. 9; Table 1). These data evidenced the multipotency of the progenitors, i.e., their capacity to fully develop into all mature cell fates for which they are precursors. Depending on the duration of differentiation conditions the content of neurons and astrocytes gradually increased (Table 1). On the other hand, the oligodendrocyte component of the culture did not change in time (Table 1). Under the differentiation conditions, fluoxetine treatment (3 and 5 days) caused a significant increase in the content of neurons compared to the corresponding vehicle-treated control. In contrast, fluoxetine treatment did not modify the glial or oligodendrocyte component of the culture (Table 1).

4. Discussion

Previous work in primary rat CGC cultures demonstrated the concentration-dependence of the effects of fluoxetine on cell proliferation. Whereas 1–2 μM fluoxetine-stimulated cell proliferation, 20 μM fluoxetine produced an opposite effect, it inhibited cell proliferation [5,6]. Recently, we established that the fluoxetine-stimulated cell proliferation in primary CGC cultures occurs in a subpopulation of round-shaped cells that bear all the hallmarks of neural progenitor cells [7]. The present study further characterized these progenitors.

Thus, upon isolation from CGC and exposure to bFGF and EGF in a serum-free medium to maintain cells in their mitotic and undifferentiated state [38], immunocytochemical analysis revealed that round-shaped cells (i.e., neural progenitors) do

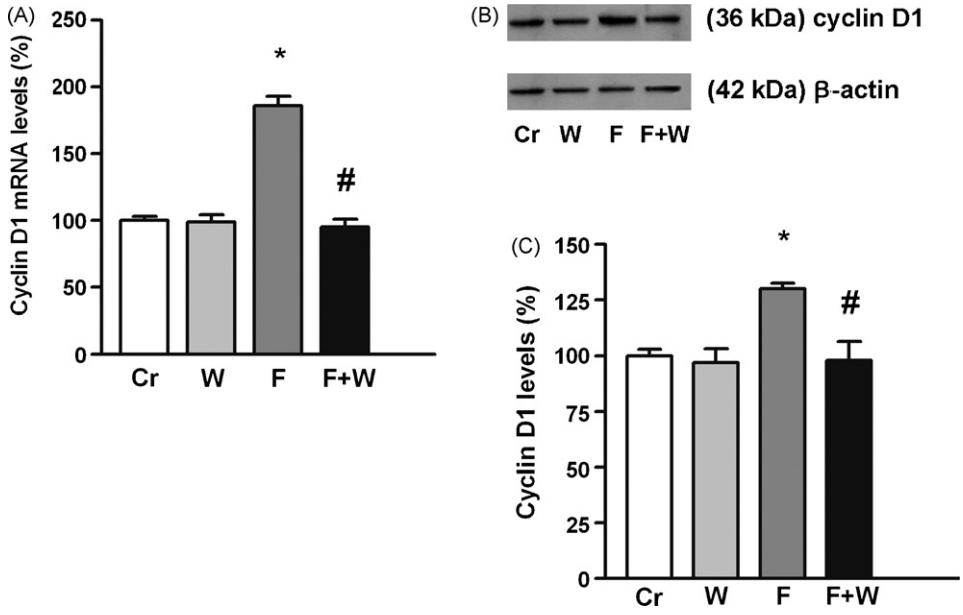

Fig. 8 – WAY-100635 prevents fluoxetine from increasing cyclin D1 mRNA and protein levels in isolated cerebellar neural progenitors. At 10 DIV, cerebellar neural progenitors maintained in *growth medium* were exposed for 48 h to vehicle (Cr) or 1 μM fluoxetine (F) in the absence or presence of 1 nM WAY-100635 (W). Cyclin D1 mRNA levels and protein content were evaluated by RT-PCR (A) and Western blotting (B) and (C), respectively. Cyclin D1 mRNA values (A) were normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Bar graph in (C) depicts the results of a densitometric quantification from immunoblots of cyclin D1 protein levels (B), normalized to β-actin levels and expressed as percentage of the vehicle-treated control. Data are the mean ± S.D. of at least 4 determinations from 3 independent experiments. $^{*}p < 0.05$ vs. control and $^{\#}p < 0.05$ vs. 1 μM fluoxetine (ANOVA and Dunnett's test for multiple comparisons).

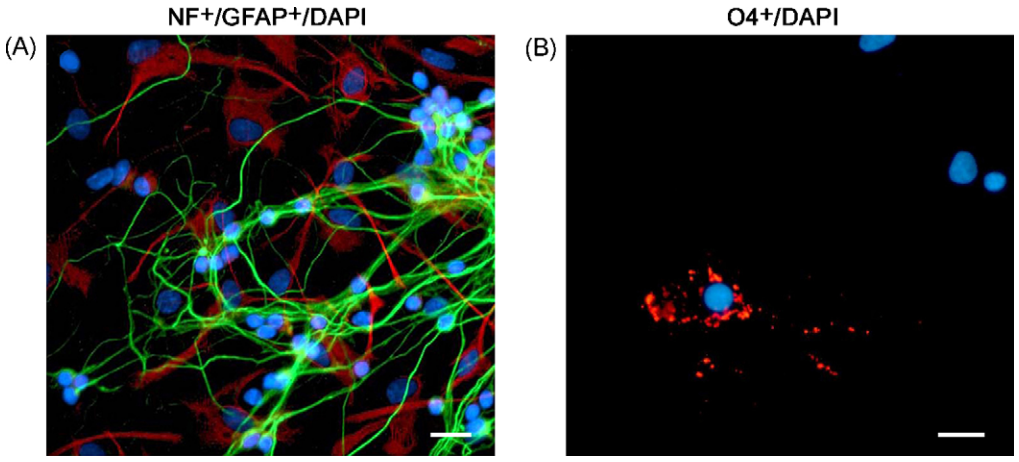

Fig. 9 – Multipotency of cerebellar neural progenitors. At 10 DIV, cells were replated onto poly-D-lysine-coated glass coverslips in the *differentiation medium* (i.e., deprived of growth factors and supplemented with 10% fetal calf serum). After 3 days, cells were fixed with 4% paraformaldehyde and double stained with specific antibodies and a nuclear stain DAPI. Panels (A) and (B) show representative fluorescent microphotographs of neurofilament-positive cells (NF^+; A, green), glial fibrillary acidic protein-positive cells ($GFAP^+$; A, red), and O4-positive cells ($O4^+$; B, red). Blue DAPI staining shows the nuclei. Scale bars = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

not exhibit immunoreactivity to markers typical of mature neuronal and glial lineages such as NF, GFAP, and O4 [7]. However, these cells are immunoreactive for several immature neuronal markers (Fig. 1). Most of them are immunoreactive for βIII-tubulin, a neuron-selective protein marking developing neurons [34]. Only a minor portion of these cells is positive for nestin and PSA-NCAM. Nestin is an intermediate filament protein which has been characterized as a marker for neuroepithelial and CNS stem and immature progenitor cells both in vitro and in vivo [32], and PSA-NCAM is typically expressed by cells in adult brain regions capable of cellular remodeling and neurogenesis [33].

We found that these progenitor cells express the key characteristics of multipotent, self-renewing neural progenitor cells [39,40]. Thus, they are capable to proliferate from single cells for several subcloning steps [7]. Further, we found that on removal of growth factors from the culture medium, they subsequently differentiate into both neurons and glial cells. Under these differentiation conditions fluoxetine treatment for up to 5 days induces significant increase in neuronal phenotypes, while leaving unchanged the glial and oligodendrocyte components. These findings demonstrated for the first time the susceptibility of isolated postnatal cerebellar neural progenitors to a selective and lasting neurogenic response to fluoxetine.

In contrast to differentiation conditions, protracted treatment of these progenitors under growth conditions with fluoxetine, 5HT (a non-selective 5HT receptor agonist), and 8-OH-PIPAT (a selective $5HT_{1A}$ receptor agonist) induces significant stimulation of cell proliferation. We found fluoxetine and 5HT to be effective only at the concentration of 1 μM, whereas 8-OH-PIPAT produces a maximum increase in cell proliferation at 100 nM. At the highest concentration tested (20 μM), all these compounds significantly decrease cell

Table 1 – Increase in neuronal differentiation of isolated cerebellar neural progenitors by 3- and 5-day fluoxetine treatment

Cell type	3 days		5 days	
	Control (%)	Fluoxetine (%)	Control (%)	Fluoxetine (%)
Neurons	7.9 ± 3.3	17.1 ± 1.2**	13.1 ± 2.2*	31.1 ± 2.1**
Astrocytes	13.1 ± 5.3	23.0 ± 6.3	39.5 ± 4.6*	38.2 ± 6.4
Oligodendrocytes	0.8 ± 0.3	0.7 ± 0.1	0.8 ± 0.2	0.6 ± 2.3

Isolated cerebellar neural progenitors at 10 DIV were replated onto poly-D-lysine-coated glass coverslips in the *differentiation medium* (i.e., deprived of growth factors and supplemented with 10% fetal calf serum). After 3- and 5-day incubation in the absence (control) or presence of 1 μM fluoxetine, cells were fixed with 4% paraformaldehyde and double stained with a nuclear stain DAPI and specific antibodies to evidence NF^+ cells (i.e., neurons), $GFAP^+$ cells (i.e., astrocytes), and 04^+ cells (i.e., oligodendrocytes). The relative numbers of neurons, astrocytes, and oligodendrocytes in control and fluoxetine-treated group were obtained from counting of NF^+, $GFAP^+$, and 04^+ cells, respectively, and were expressed as percentages of the total DAPI-stained cell population. Data are the mean ± S.D. of nine coverslips/group from 3 independent experiments (ANOVA and Bonferroni's test for selected comparisons).

* $p < 0.05$ vs. 3 days control.

** $p < 0.05$ vs. corresponding vehicle-treated control.

proliferation, probably as a consequence of their cytotoxicity. The concentration dependency of the proliferative fluoxetine action is in agreement with previous findings in primary rat CGC cultures [5,6], and the low micromolar concentration of fluoxetine used in our present study is relevant to the therapeutic plasma concentration of this drug [41].

The proliferative action of the serotonergic compounds used in our study is completely abolished by the selective $5HT_{1A}$ receptor antagonist WAY-100635, suggesting a crucial role of these receptors. The phenotypic characterization of cerebellar neural progenitor cells by RT-PCR shows that they express mRNAs for TPH, SERT, and several serotonergic receptor subtypes, including $5HT_{1A}$. Furthermore, our immunocytochemical assays confirmed that these cells not only express SERT and $5HT_{1A}$ receptors, but also 5HT and TPH, thus indicating that they are capable of synthesizing 5HT. Interestingly, fluoxetine treatment decreases the intensity of 5HT-immunoreactivity, i.e., the cellular 5HT content, while leaving unchanged the percentage of cells immunopositive for 5HT. These data suggest a reduction in 5HT intracellular levels, which could occur due to inability of fluoxetine-treated cells to take up serotonin via the SERT, which is inhibited by fluoxetine.

It was unexpected to find that postnatal cerebellar neural progenitors express all the above-noted serotonergic phenotypes including the $5HT_{1A}$ receptor-dependent responsiveness to fluoxetine, since the mature cerebellum is devoid of intrinsic serotonergic neurons and significant amounts of $5HT_{1A}$ receptors [42,43]. However, it is known that 5HT projections are critical to cerebellar function and early transient dense expression of 5HT receptors ($5HT_{1A}$) appears to be important for the formation of the cerebellar cortex [14]. Furthermore, it has been noted that cerebellar $5HT_{1A}$ receptors undergo pronounced developmental changes in both rodent and human cerebellum; their expression is abundant in neonates but almost absent in adults [42,44]. In immature rat cerebellum, e.g., at postnatal day 11, $5HT_{1A}$ receptors are found in Purkinje cells, the external granule cell layer (i.e., the region of neural progenitors), and in the internal granule cell layer [45].

Our data suggest that $5HT_{1A}$ receptors render cerebellar neural progenitors susceptible to the proliferative action of fluoxetine. Thus, it appears that the postnatal period of an active and proliferating external granule layer could make the infant cerebellum susceptible to these actions of fluoxetine and possibly other SSRIs. Significantly, human cerebellum may be susceptible to these actions for much longer period of time; i.e. 11 months [8] compared to rodent cerebellum (21–22 days) [46,47]. Recently, cerebellum has attracted the attention of autism researchers who suggested that cerebellum might be involved due to its role in attention regulation and motor control [14]. Interestingly, pharmacological agents affecting the 5HT system, including the SSRIs, appear to be useful in improving symptoms in individuals with autism [13–15].

More generally, our present findings are in agreement with the notion that 5HT exerts trophic effects both during CNS development [48] and also in adulthood [49,50], possibly via activation of brain $5HT_{1A}$ receptors. Thus, recent observations made in the adult hippocampus have shown that $5HT_{1A}$ receptor activation is required during either naturally occurring proliferation [18,51] or fluoxetine-induced cell proliferation, survival, and differentiation [22].

The intracellular signaling pathways and networks linking upstream receptor activation to the downstream cellular responses of neural progenitors have been poorly defined. Several lines of investigation have demonstrated that in vivo chronic but not acute administration of various antidepressants increases the mRNA and protein levels of the transcription factor CREB and the neurotrophin BDNF in the adult hippocampus [20,24–26,52,53]. Moreover, antidepressants that increase neurogenesis typically modulate the expression, phosphorylation, and transcriptional activity of CREB [25,26], which may mediate the downstream induction of BDNF gene expression [24,25,27]. The transcriptional activity of CREB is induced by its phosphorylation on the residue Ser^{133} by a number of protein kinases, including cAMP-dependent protein kinase (PKA), calcium/calmodulin (CaM)-dependent protein kinases, and members of the ERK/MAPK kinase cascades [25,26,54]. Since the gene transcription efficiency of CREB is correlated with the duration of Ser^{133} phosphorylation, activation of the ERK/MAPK kinase cascades may have a primary role in the regulation of neuronal CREB function by inducing the necessary sustained CREB phosphorylation [29].

In line with the findings obtained mainly in the hippocampus and cortex [25,26,54], we found that protracted fluoxetine treatment of cerebellar neural progenitors cultured under growth conditions (i.e., in medium containing growth factors) significantly increased CREB phosphorylation and activated ERK1/2. These effects are not due to a change in total levels of CREB and ERK1/2 proteins, pointing to modulation of phosphorylation rather than altered expression as a main target in the action of fluoxetine. The increase in both pCREB and pERK1/2 levels is completely blocked by WAY-100635, suggesting the involvement of $5\text{-}HT_{1A}$ receptors, which is consistent with the findings by other investigators [26]. In addition, blocking the activation of the ERK/MAPK kinase cascades with the selective MEK1/2 inhibitor U0126 [37] completely prevents both the downstream increase in pERK1/2 and cell proliferation induced by protracted fluoxetine treatment. These findings suggest that the proliferative response of cerebellar neural progenitors to fluoxetine is entirely supported by a full efficiency of the MAPK pathway. U0126 alone also decreases the basal levels of both pERK1/2 and proliferation in control cells, suggesting that the activation of the ERK/MAPK kinase cascades mediates the proliferative activity of growth factors [55] present in the culture medium.

Taken together these findings further support the view that the $5HT_{1A}$ receptor-mediated activation of ERK/MAPK kinase cascades may represent an important component in the fluoxetine-induced cellular responses in cerebellar neural progenitors. Although the $5HT_{1A}$ receptor is a G_i/G_o-protein-coupled receptor, it has been shown that, similarly to other G-protein-coupled receptors, its activation increases the phosphorylation of ERK, both in vitro [56,57] and in vivo [58], possibly through a G_i protein $\beta\gamma$-subunit-mediated pathway that involves many of the same molecules used by growth factor receptor tyrosine kinases [59].

The observation that the ERK/MAPK signaling pathway, activated by growth factors, may interact with serotonergic

receptors in regulating cell proliferation by upregulating the expression of cyclin D1, which in turn is essential for the cell cycle progression [30,58,60], has prompted us to examine whether protracted fluoxetine treatment of cerebellar neural progenitors affects cyclin D1. We found a significant increase in both cyclin D1 mRNA and protein levels that is counteracted by the $5HT_{1A}$ antagonist WAY-100635. These data are similar to a previously noted role of $5HT_{2B}$ receptors in the regulation of cyclin D1 and mitogenesis [60] and require further research to fully elucidate the interplay between various 5HT receptor subtypes [59] in regulating neural progenitors.

Regarding BDNF, we did not find changes either in BDNF mRNA levels or protein expression in fluoxetine-treated cerebellar neural progenitors maintained under growth conditions (data not shown). It has been noted that the stimulatory action of antidepressants on BDNF is variable and may not be observed under every experimental condition. Thus, some authors observed the increased expression of BDNF mRNA [52,61,62], while others show no change in BDNF exon-specific transcripts [63] or protein [64], and even decreased BDNF mRNA [65]. An alternative explanation is that in cerebellar neural progenitors maintained under growth conditions, the $5HT_{1A}$ receptor activation induced by fluoxetine treatment transactivates receptor tyrosine kinases TrkB BDNF receptors. This could occur without altering BDNF content and would affect the downstream ERK signaling cascade, as it has been recently reported for certain G-protein-coupled receptor ligands [66,67].

In summary, our results provide clear evidence that early postnatal rat cerebellum contains multipotent neural progenitor cells capable of proliferating and differentiating in response to prolonged fluoxetine treatment. The ERK/MAPK kinase pathway is the likely target through which fluoxetine acts to promote higher pCREB levels and an increased expression of cyclin D1. It appears that these effects occur through the activation of $5HT_{1A}$ receptors, as they are counteracted by the $5HT_{1A}$ receptor antagonist WAY-100635.

Although no data are available on whether fluoxetine affects quiescent cerebellar neural progenitors in vivo, our results may be clinically relevant because these progenitors are active in the human cerebellum up to 11 months postnatally [8] and fluoxetine and its active metabolite norfluoxetine can accumulate in nursing infants via breastfeeding [9]. In addition to their unintentional administration to infants, SSRIs including fluoxetine are widely used as a treatment for infantile autism [13–15]. Therefore, an increased drug-induced proliferation and/or differentiation of neural progenitors in the cerebellum of infants might result in long-term behavioral changes. Further studies are needed to fully clarify the in vivo implications of our in vitro studies.

Acknowledgements

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