

# Serotonin (5-HT) Regulates Neurite Outgrowth Through 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> Receptors in Cultured Hippocampal Neurons

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Serotonin (5-HT) production and expression of 5-HT receptors (5-HTRs) occur early during prenatal development. Recent evidence suggests that, in addition to its classical role as a neurotransmitter, 5-HT regulates neuronal connectivity during mammalian development by modulating cell migration and neuronal cytoarchitecture. Given the variety of 5-HTRs, researchers have had difficulty clarifying the specific role of each receptor subtype in brain development. Signalling mediated by the G-protein-coupled 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R, however, has been associated with neuronal plasticity. Thus, we hypothesized that 5-HT promotes neurite outgrowth through 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R. The involvement of 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R in the morphology of rat hippocampal neurons was evaluated by treating primary cultures at 2 days *in vitro* with 5-HT and specific antagonists for 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R (WAY-100635 and SB269970, respectively). The stimulation of hippocampal neurons with 100 nM 5-HT for 24 hr produced no effect on either the number or the length of primary neurites. Nonetheless, after 5HT<sub>7</sub>R was blocked, the addition of 5-HT increased the number of primary neurites, suggesting that 5HT<sub>7</sub>R could inhibit neurite outgrowth. In contrast, 5-HT induced secondary neurite outgrowth, an effect inhibited by 1 μM WAY-100635 or SB269970. These results suggest that both serotonergic receptors participate in secondary neurite outgrowth. We conclude that 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R regulate neuronal morphology in primary hippocampal cultures by promoting secondary neurite outgrowth. © 2014 Wiley Periodicals, Inc.

**Key words:** serotonin; 5-HT<sub>1A</sub> receptor; 5-HT<sub>7</sub> receptor; outgrowth; neurites; hippocampus; neuron; culture

Serotonin (5-HT) is one of the first neurotransmitters to be expressed in the developing brain and performs important functions during brain development (Gaspar et al., 2003). By embryonic day 12 (E12), serotonergic neurons can be identified in the rat brainstem. The

onset of profusely branched axons projecting from the brainstem to different areas of the brain coincides with neuronal growth and differentiation (Wallace and Lauder, 1983). The caudal portion of the brainstem (represented by the B1–B5 nuclei) corresponds to raphe pallidus, obscurus, and pontis; the rostral portion of the brainstem (represented by the B6–B9 nuclei) corresponds to raphe dorsal and medial (Jacobs and Azmitia, 1992).

5-HT may be involved in several aspects of neuronal development, including dendritic growth and complexity (van Kesteren and Spencer, 2003). Pharmacological depletion of 5-HT during E12–17 inhibits the growth and dendritic arborization of cortical pyramidal neurons, suggesting that 5-HT may promote both outgrowth and branching of dendrites in these neurons (Vitalis et al., 2007). Moreover, a decrease in dendritic arborization of cortical neurons has been shown in animals maintained on a diet low in tryptophan (Gonzalez-Burgos et al., 1996), an essential amino acid required for synthesis of 5-HT. Additionally, the depletion of 5-HT early on postnatal day 3 (P3) reduces dendritic length and spine density in hippocampal granule neurons (Yan et al., 1997b).

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Thus, 5-HT can be considered a trophic agent for promoting outgrowth and arborization of dendrites during brain development. Moreover, the effect of 5-HT on neuronal morphology may be mediated through the activation of specific 5-HT receptors expressed at appropriate stages of brain development.

5-HT activates a large family of G-protein-coupled receptors in the central and peripheral nervous systems. Seven 5-HTR subtypes are coupled to G $\alpha$ -proteins (Mattson et al., 2004). 5-HT<sub>1</sub>R is coupled to  $\alpha_i/\alpha_o$ ; 5-HT<sub>2</sub>R is coupled to  $\alpha_q$ ; and the receptor subtypes 5-HT<sub>4</sub>, 5-HT<sub>5</sub>, 5-HT<sub>6</sub>, and 5-HT<sub>7</sub> are coupled to  $\alpha_s$  (Albert and Tiberi, 2001). Studies have shown that some 5-HT receptor subtypes are expressed early in embryonic life and are dynamically regulated during pre- and postnatal development (reviewed by Gaspar et al., 2003). Remarkably, hippocampal neurons rapidly initiate expression of 5-HT<sub>1A</sub>R protein by E16 (Patel and Zhou, 2005), before the arrival of serotonergic axons at E19 (Lidov and Molliver, 1982). There is some evidence that 5-HT<sub>1A</sub>R can regulate neuronal morphology. For instance, juvenile and adult Htr1a knockout mice show increased arborization of hippocampal pyramidal neurons, accompanied by anxious behavior (Cumbo et al., 2008). Moreover, these morphological changes are also observed in the pharmacological blockade of 5-HT<sub>1A</sub>R during the third to fifth postnatal weeks of mice (Ferreira et al., 2010). In addition, reduced dendritic length and spine density in hippocampal granular neurons are observed after postnatal depletion of 5-HT (Yan et al., 1997a). This reduction is prevented by administration of a 5-HT<sub>1A</sub>R agonist (Yan et al., 1997a). These findings suggest that 5-HT<sub>1A</sub>R plays a pivotal role in hippocampal neuronal morphology.

In contrast, it has been demonstrated that activation of 5-HT<sub>7</sub>R stimulates synaptogenesis in hippocampal neurons at early postnatal stages (Kobe et al., 2012). Moreover, studies of 5-HT<sub>7</sub>R in fibroblast cell lines have established a relationship between this receptor and proteins controlling the neuronal cytoskeleton, such as RhoA, Cdc42, and G $\alpha$ 12 (Kvachnina et al., 2005). These data suggest that 5-HT<sub>7</sub>R may play a critical role in regulating neuronal cytoarchitecture. More recently, it has been shown that coordinated variations in levels of 5-HT<sub>1A</sub> and 5-HT<sub>7</sub>R occur during early postnatal development of the hippocampus (Renner et al., 2012), suggesting that these receptors may contribute differentially to the formation of the hippocampal neurocircuitry.

We hypothesized that 5-HT promotes neurite outgrowth through 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R. To test this hypothesis, we investigated the effects of 5-HT and 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R antagonists on neurite outgrowth in primary cultures of rat hippocampal neurons. We showed that activation of 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R in primary hippocampal cultures promoted neurite outgrowth, suggesting that alterations in these receptors may produce detrimental effects on the wiring of the brain during development, leading to an increased susceptibility to various mental disorders.

## MATERIALS AND METHODS

### Hippocampal Culture

All procedures were approved by the Ethical Committee of the Faculty of Chemical and Pharmaceutical Sciences, Universidad de Chile, and were in compliance with the National Institutes of Health Guide for the care and use of laboratory animals. Primary hippocampal neurons were cultured as previously described by Fiedler et al. (1994), with some modifications. E18 embryos were removed by caesarean section from anesthetized, pregnant Sprague-Dawley mothers. Brains from the embryos were dissected and immediately placed in Hank's balanced salt solution (1 mM Hepes, 140 mM NaCl, 5 mM KCl, 5 mM glucose, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>) supplemented with 1,000 IU/ml penicillin-streptomycin (Gibco, Life Technologies, Grand Island, NY). Rat hippocampi were dissected and incubated for 15 min in Hank's balanced solution with 0.05% trypsin (Biological Industries, Beit HaEmek, Israel). Enzymatic digestion was stopped by adding 1 ml Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (Hyclone, Logan, Utah), 2 mM GlutaMAX (Life Technologies), 1 mM sodium pyruvate (Sigma-Aldrich), and 1,000 IU/ml penicillin-streptomycin (Life Technologies). Digests were then centrifuged at 235 g for 4 min. The pelleted cells were mechanically dissociated with a Pasteur pipette in DMEM supplemented with 10% fetal bovine serum. For immunocytochemistry studies, cells were plated on 35-mm coverslips precoated with poly-D-lysine (0.1 mg/ml) at a density of 80,000 cells/well. After 2 hr in the presence of 5% CO<sub>2</sub> at 37°C, DMEM was replaced with an equal volume of neurobasal medium supplemented with 1% v/v B27, 2 mM GlutaMAX, 1 mM sodium pyruvate, and 1,000 IU/ml penicillin-streptomycin. Neurobasal medium supplemented with B27 favors neuronal development and limits astrocyte proliferation.

### Pharmacological Treatments

To determine the effect of serotonergic receptor activation on neuron morphology, 5-HT, WAY-100635 (Sigma-Aldrich), and SB269970 (Tocris Bioscience, Ellisville, MO) were dissolved in neurobasal medium and added to cells at 2 DIV. Cells were stimulated for 24 hr with 5-HT. To detect the contribution of 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R, antagonists were added 30 min prior to addition of agonist.

### Reverse Transcriptase Coupled to Quantitative Real-Time Polymerase Chain Reaction

Hippocampal primary cultures (10<sup>6</sup> cells) were lysed in Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and the RNA integrity was evaluated by using denaturing gel electrophoresis. Total RNA (2,500 ng) was reverse transcribed into cDNA by using Superscript II (Invitrogen) and 250 ng of random primers (Invitrogen) following the manufacturer's protocol. Quantitative real-time polymerase chain reaction (RT-PCR) experiments were conducted on a Stratagene Mx3000p thermocycler (Agilent, Santa Clara, CA) programmed as follows: 95°C for 10 min followed by 40

cycles of 95°C for 15 sec, 60°C for 15 sec, and 70°C for 20 sec. Each reaction was carried out in duplicate with 10 µl Brilliant II Ultra-Fast SYBR Green QPCR Master Mix (Agilent), the appropriate dilution of RT, and 0.12 µM of the forward and reverse primers. Primers were designed by using Primer-Blast (NCBI) and obtained from Integrated DNA Technologies (Coralville, IA). Primers for 5-HT<sub>1A</sub>R correspond to a unique exon sequence based on NM\_012585.1 (forward 5'-GGCTATCACCGACCCTATAGAC-3', reverse 5'-AAGA TGCGCCCGTAGAGAAC-3') and generate a fragment of 249 bp. Primers for 5-HT<sub>7</sub>R correspond to a unique exon sequence based on NM\_022938.2 (forward 5'-ATTTACAAGGCCGCC AGGAA-3', reverse 5'-CTCCCACGATGATCC CCAAG-3') and generate a fragment of 220 bp. The relative abundance of these targets was normalized to levels of β-actin with the following primers: forward 5'-TTGTCCCTGTATGCCTC TGGTC-3', reverse 5'-ACCGCTCATTGCCGATAGTG-3', generating a fragment of 346 bp. Standard curves for all primer sets were conducted with serial dilutions of cDNAs, and specificity was validated by using melting curve analyses. Relative gene mRNA level was calculated based on the ΔΔCt for each mRNA, normalized to that of the housekeeping gene mRNA.

Additionally, conventional RT-PCR was conducted with a dilution of the RT reaction, with 1 U Go TaqFlexi DNA polymerase (Promega, Madison, WI), 0.5 mM dNTPs, and 0.5 µM of each primer, in a final volume of 30 µl. PCR was conducted for 28 cycles for 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R and 18 cycles for β-actin. PCR consisted of denaturation at 92°C for 30 sec followed by annealing at 59°C for 30 sec and extension at 72°C for 30 sec by using a Techne TC-512 DNA thermal cycler (Bibby Scientific, Staffordshire, United Kingdom). All RT-PCRs and PCRs included the use of water in place of template as a negative control and input of RNA without RT reaction. RT-PCR products were electrophoresed in 1.5% agarose gels, stained with GelRed nucleic acid stain (Biotium, Hayward, CA), and photographed.

### Protein Extraction and Western Blotting

Cells were lysed in the presence of 25 mM Tris (pH 7.4), 75 mM NaCl, 0.5 mM EGTA, 0.5 mM EDTA, 0.4 mM DTT, 0.125 mM Na<sub>3</sub>VO<sub>4</sub>, 0.2 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 2 mM NaF, 2 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, and 1% Triton X-100; then sonicated on ice for 5 min. After centrifugation of lysates at 17,860 g for 30 min, the supernatant was collected, and a sample was saved for protein determination by using the bicinchoninic method (Sapan et al., 1999). The remaining supernatant was boiled immediately in sample loading buffer. In total, 15 µg of each protein extract was resolved on 12% SDS-polyacrylamide gels and then blotted onto a 0.2-µm nitrocellulose membrane. Membranes were blocked in 3% nonfat milk dissolved in TBS with 0.1% Tween (TBS-T). Membranes were incubated overnight in a 1:500 dilution of anti-5-HT<sub>1A</sub> antibody (H-119; Santa Cruz Biotechnology, Santa Cruz, CA) or a 1:250 dilution of anti-5-HT<sub>7</sub> antibody (Ab13898; Abcam, Cambridge, MA). After rinsing the membranes in TBS-T, blots were incubated with peroxidase-conjugated anti-rabbit secondary antibody (1:10,000, anti-rabbit affinity-purified; Thermo Fisher Scientific, Waltham, MA) at room temperature for 2 hr. Membranes were then incubated with enhanced chemilumi-

nescent (ECL) substrate (Biological Industries). After stripping, membranes were incubated overnight in a 1:10,000 dilution of anti-β-actin antibody (Sigma-Aldrich). Membranes were then incubated with peroxidase-conjugated anti-rabbit secondary antibody and processed as described above. Band intensities were determined in the UNSCAN-IT program (Silk Scientific, Orem, UT). Data are expressed as the ratio between the band intensity of 5-HT<sub>1A</sub>R or 5-HT<sub>7</sub>R and that of β-actin.

### Immunocytochemistry

Cells grown on coverslips were fixed and permeabilized at 3 DIV in accordance with methods published by Witte et al. (2008). To study neuronal morphology, a 1:20,000 dilution of antiacetylated tubulin antibody (Sigma-Aldrich) followed by incubation with a 1:300 dilution of anti-mouse Alexa Fluor 568 (Molecular Probes, Eugene, OR) was used to label microtubules. Coverslips were then rinsed in PBS and incubated with a 1:500 dilution of rhodamine-phalloidin (Invitrogen) to label actin filaments. Nuclei were then stained with 0.5 µg/ml Hoechst 33342 (Molecular Probes). Finally, cells were washed and mounted on glass slides with fluorescence-preserving medium (Dako Mounting Medium; Dako, Carpinteria, CA).

### Image Analysis

Images were obtained with an IX81 disk scanning unit microscope (Olympus, Tokyo, Japan) at ×40 magnification. Image J software (NIH, rsb.info.nih.gov/ij) was used to merge the fluorescent signals from three fluorophores into a single image. Nuclear staining (blue signal) was used to verify that the signals originated from a single neuron. Actin staining (red signal) was used to observe the growth cone and approximate the length of the projections. Tubulin staining (green signal) was used to visualise neurites. Numbers of neurites and mean neurite length were determined for each individual hippocampal neuron in NeuronJ software. The numbers of primary and secondary neurites and the mean neurite lengths were also counted.

Primary neurites were defined as processes extending directly from the neuronal soma, and secondary neurites were defined as processes extending from a primary neurite. We have observed that at low neuron plating densities the growth during DIV 3–8 is associated with secondary neurites (not shown). We therefore reasoned that stimuli probably have the largest effect on the most plastic neurites (i.e., those with low length). Thus, we decided to segregate primary and secondary neurites according to their length, with a cutoff based on the mean value obtained from control neurons (mean value for primary 39.58 ± 1.66 µm, mean value for secondary 17.11 ± 1.44 µm). We used these criteria to segregate the neurites according to length; short (≤40 µm) and long (>40 µm) primary neurites and short (≤20 µm) and long (>20 µm) secondary neurites.

To conduct a double-blind analysis, the following precautions were taken. Images were renamed randomly in the program BlindTreatment.jar (Popko et al., 2009), which was generously provided by Dr. Lorene Lanier (University of Minnesota). Neuronal imaging and quantitation were performed by different investigators. After tracing the projections in NeuronJ, we exported the raw data from NeuronJ to Microsoft Excel



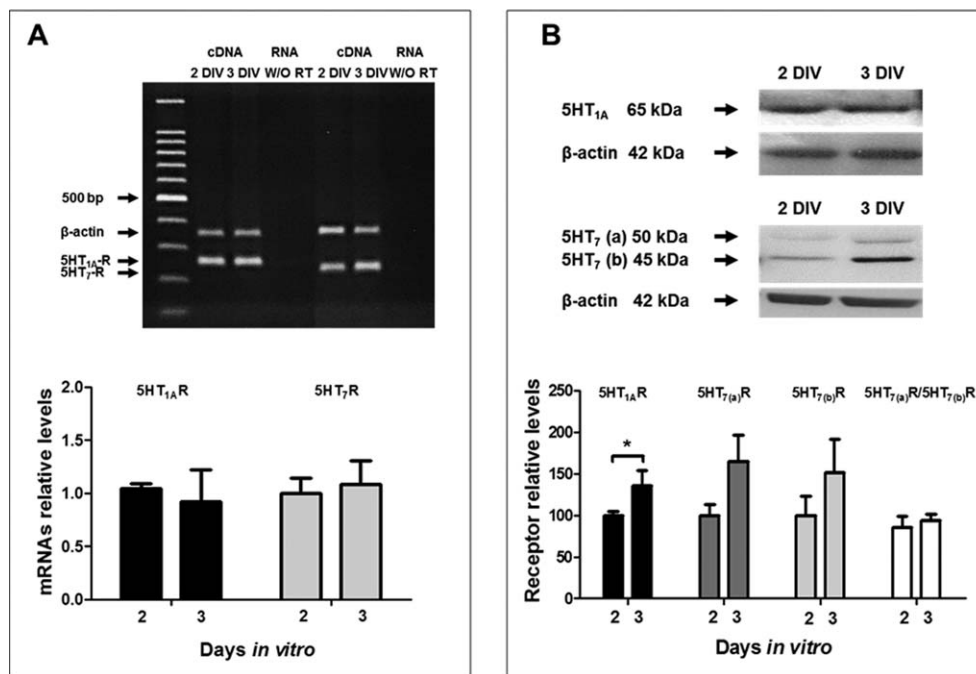


Fig. 1. Variation of 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R mRNAs and protein levels from 2 to 3 DIV. **A:** Detection of 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R mRNAs in hippocampal cultures at 2 and 3 DIV. The specific mRNAs were detected by conventional RT-PCR (upper panel) and real-time qPCR (lower panel) with  $\beta$ -actin as a housekeeping gene. Primers for 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R mRNAs generate products of 249 bp and 220 bp, respectively. The upper panel shows the products obtained by conventional RT-PCR. Column 1, 100-bp standard; columns 2 and 3 show that 5-HT<sub>1A</sub>R is detected in cultures at 2 and 3 DIV; column 4, RNA without RT; columns 5 and 6 show that 5-HT<sub>7</sub>R is detected in cultures at 2 and 3 DIV; column 7, RNA without RT. The lower panel shows the relative mean mRNA expression

level of 5-HT<sub>1A</sub> and 5-HT<sub>7</sub>R mRNAs in cultures at 2 and 3 DIV obtained by qPCR ( $n = 3-4$ ). **B:** Representative western blots showing the levels of 5-HT<sub>1A</sub>R, 5-HT<sub>7</sub>R, and  $\beta$ -actin in extracts from cultures at 2 and 3 DIV. The anti-5-HT<sub>1A</sub>R antibody detected one band of approximately 65 kDa. The anti-5-HT<sub>7</sub>R antibody detected two bands of approximately 50 (5-HT<sub>7a</sub>R) and 45 kDa (5-HT<sub>7b</sub>R). **C:** Variation of 5-HT<sub>1A</sub>R, 5-HT<sub>7a</sub>R and 5-HT<sub>7b</sub>R levels during the evolution of the culture and analysis of the ratio between 5-HT<sub>7a</sub>R and 5-HT<sub>7b</sub>R receptor isoforms ( $n = 5$ ). Relative protein levels were determined using densitometry with  $\beta$ -actin as a loading control. The data represent mean  $\pm$  SEM normalized against the levels observed at 2 DIV. qPCR, quantitative polymerase chain reaction. The data were analyzed using Mann-Whitney U test. \* $P < 0.05$ .

and determined the variation in the length and the number of neurites. Additionally, a Sholl analysis (Sholl, 1953) was conducted on these data. A Sholl analysis uses a "skeleton" of the original image to determine the number of neurites through concentric rings that move outward from the soma in increments of 10  $\mu$ m. For the Sholl analysis, we used Bonfire 1.0 (Bonnie Firestein Lab Sholl Analysis Software, Rutgers University), software developed for Matlab 7.0 (MathWorks, Natick, MA; Langhammer et al., 2010). NeuronStudio 0.9.92 software (CNIC, Mount Sinai School of Medicine) was used to quantify branching points and ending points.

### Statistical Analysis

Statistical significance of differences between densitometric analyses of Western blots was assessed by one-tailed Mann-Whitney U tests. A nonparametric analysis was used for Western blot analyses because the number of determinations was too low to conduct a normality test (Kolmogorov-Smirnov). For morphological analyses, data passed the normality test; for analyses of two groups, we used Student's *t*-test, and, for analyses of

multiple groups ( $n > 2$ ), one-way ANOVA with the Bonferroni post hoc test was used. GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA) was used for statistical analyses. The experimental number,  $n$ , corresponds to the total number of neurons ( $n = 35-50$ ) analyzed from independent cultures conducted in duplicate.

## RESULTS

### Variation of Both mRNA and Protein Levels of 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R From 2 to 3 DIV

To evaluate the effect of 5-HTRs on the morphology of hippocampal pyramidal neurons, we first determined the levels of mRNAs for 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R at 2 and 3 DIV in total RNA (Fig. 1A). With conventional RT-PCR, mRNAs for 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R were detected both at 2 and 3 DIV (Fig. 1A). Quantitative PCR analyses of hippocampal cultures showed that the levels of both mRNAs were unchanged from 2 to 3 DIV (Fig. 1A). Additionally, extracts of hippocampal neurons were analyzed by Western blot. Figure 1B shows one

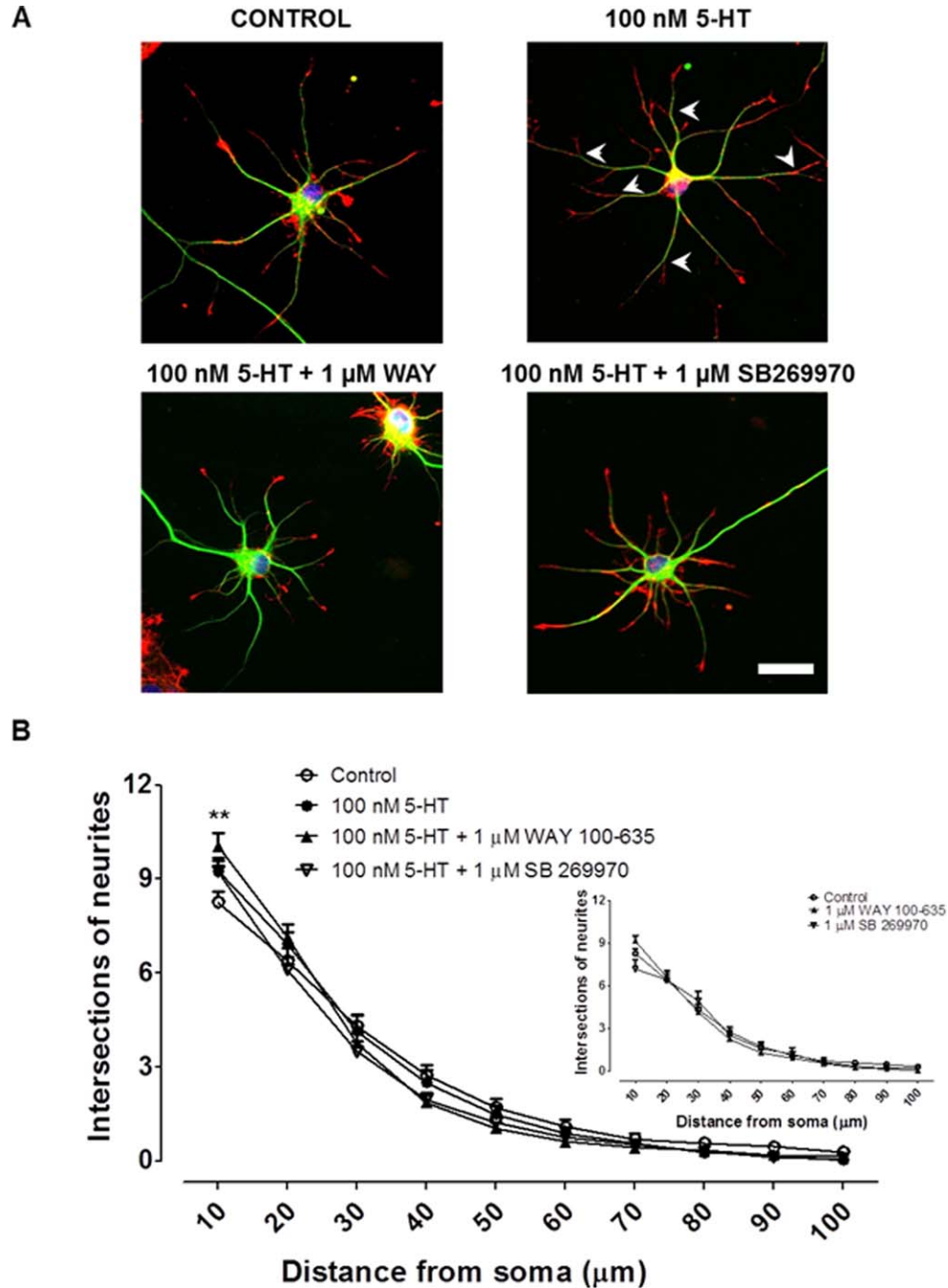


Fig. 2. Representative images of neurons used for morphometric analyses and the effect of 5-HT on neuronal morphology of hippocampal primary cultures. **A:** Neurons were cultured for 2 days and then stimulated for 24 hr with 5-HT. The antagonists WAY-100635 and SB269970 were added 30 min prior to 5-HT. At 3 DIV, cells were fixed and immunostained for acetylated tubulin (green fluorescence) and stained with rhodamine-phalloidin (F-actin marker, red fluorescence) and with nuclear staining with Hoechst dye (blue fluorescence). A disk scanning unit microscope with a  $\times 40$  objective was used, and channels were merged in ImageJ. Representative micrographs of untreated neurons (control) or neurons treated for 24 hr with 100 nM 5-HT, 100 nM 5-HT + 1  $\mu$ M WAY-100635, or 100 nM 5-HT + 1  $\mu$ M SB269970. It is apparent that 5HT does not affect primary dendrites but seems to change the length of secondary neurites. Arrowheads show secondary neurites. **B:** Sholl analysis shows the number of intersections of neurites as a func-

tion of path length from the soma. Cultures were incubated for 24 hr with 100 nM 5-HT. The antagonists WAY-100635 and SB269970 were added 30 min prior to 5-HT. Neurons were fixed and immunostained with antiacetylated tubulin antibody. F-actin was stained with rhodamine-phalloidin. The number of intersections was determined in Bonfire. Analyzed neurons from independent cultures: control ( $n = 35$ ), 5-HT ( $n = 39$ ), 100 nM 5-HT + 1  $\mu$ M WAY-100635 ( $n = 51$ ), and 100 nM 5-HT + 1  $\mu$ M SB269970 ( $n = 51$ ). These neurons were processed for various parameters, as shown in Figures 3–5. The data represent the mean  $\pm$  SEM. The inset shows the effects of antagonists alone on neuronal complexity. The data were analyzed by ANOVA for each condition followed by the Bonferroni post hoc test. Post hoc test, control vs. 5-HT + WAY-100635. ANOVA analysis,  $**P < 0.01$ . Scale bar = 50  $\mu$ m. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

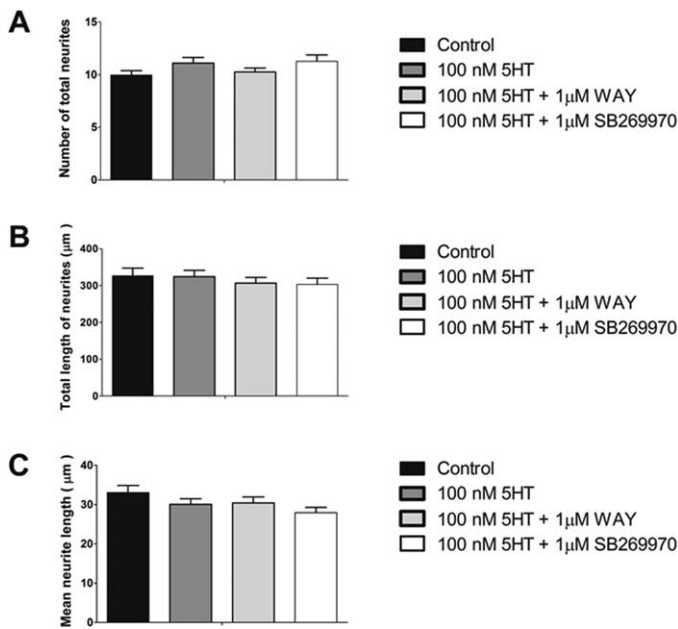


Fig. 3. Effect of 5-HT on the number and the total length of neurites. Cultures were incubated for 24 hr with 100 nM 5-HT. The antagonists WAY-100635 and SB269970 were added 30 min prior to 5-HT. Number (A), total length (B), and mean length (C) of neurites were determined for each individual hippocampal neuron in NeuronJ software. The data represent the mean  $\pm$  SEM. The data were analyzed using ANOVA for each condition followed by the Bonferroni post hoc test.

band for 5-HT<sub>1A</sub>R (65 kDa) and two bands for 5-HT<sub>7</sub>R (45 and 50 kDa for 5-HT<sub>7a</sub>R and 5-HT<sub>7b</sub>R isoforms, respectively; Ruat et al., 1993). At 3 DIV, the level of 5-HT<sub>1A</sub>R increased significantly (36%) from the level at 2 DIV (Fig. 1B). Moreover, the protein levels for 5HT<sub>7a</sub>R and 5HT<sub>7b</sub>R showed an increasing trend ( $P=0.06$  for 5HT<sub>7a</sub>R), although it was not significant (Fig. 1B). The ratio of the two 5-HT<sub>7</sub>R isoforms was constant between 2 and 3 DIV (Fig. 1B), suggesting that the expression of both isoforms could be regulated in the same direction in culture.

### Effects of 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R on Neuronal Morphology

To determine the effect of 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R on neurite outgrowth, hippocampal neurons were preincubated for 30 min with no treatment or with 1  $\mu$ M WAY-100635 or 1  $\mu$ M SB269970. Neurons were subsequently stimulated with 100 nM 5-HT for 24 hr. Representative images of neurons at 3 DIV after 24 hr of incubation with 5-HT in the presence and absence of the antagonist are shown in Figure 2A. Stable microtubules were visualized by immunolabeling acetylated tubulin in neurons. Axons were not included in the analyses of neurites. Qualitative analyses show that stimulation with 5-HT does not affect primary dendrites but seems to promote variations in the length of secondary neurites. The

effect of 5-HT on secondary neurites seems to be prevented by the antagonists WAY-100635 and SB269970 (Fig. 2A). We used a Sholl analysis to quantitate changes in neuronal morphology, including ramification and branching points. Compared with untreated controls, stimulation with 5-HT in the presence or absence of 5-HT<sub>1A</sub>R or 5-HT<sub>7</sub>R antagonists had no significant effect on neurite complexity within a 10–100- $\mu$ m radius from the soma (Fig. 2B). Moreover, no effects were observed upon treatment with WAY-100635 or SB 269970 alone (Fig. 2, inset). After 5-HT<sub>1A</sub>R was blocked, the addition of 5-HT for 24 hr slightly increased the number of intersections (Fig. 2B) within a 10- $\mu$ m radius from the soma, suggesting that another 5-HTR could promote changes in the number of primary neurites (Fig. 2B). The blocking of 5-HT<sub>7</sub>R produced a similar effect, but it was not statistically significant.

The effects of 5-HT and 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R on neuron morphology and number, total neurite length, and mean neurite length were analyzed quantitatively. Figure 3 shows no clear differences in neurite number or either total or mean neurite length after these treatments. However, this type of analysis probably is limited to the effect of 5-HT and the specific activation of 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R on a specific population of neurites. With this in mind, to verify whether these treatments promote changes in neurite parameters, we decided to reevaluate the data considering primary and secondary neurites separately. Additionally, we have observed that, at low plating densities, the growth during DIV 3–8 is associated with secondary neurites (not shown). We therefore reasoned that the stimulus is likely to have the largest effect on the most plastic neurites, i.e., neurites with low length, which are more sensitive to external stimuli. The neurites were segregated in length as short ( $\leq 40$   $\mu$ m) and long ( $> 40$   $\mu$ m) primary neurites and short ( $\leq 20$   $\mu$ m) and long ( $> 20$   $\mu$ m) secondary neurites. Figure 4 shows that the incubation with 5-HT produced no significant changes in either the number (Fig. 4A, left) or the mean length of primary neurites (Fig. 4B, left). Nonetheless, after 5HT<sub>7</sub>R was blocked, the addition of 5-HT for 24 hr significantly increased the number of primary neurites (Fig. 4A, left). This effect was based on a significant increase in short primary neurites, accompanied by a reduction in the number of long primary neurites, although the latter effect was not statistically significant (Fig. 4A, right). Furthermore, after the selective blockade of 5-HT<sub>7</sub>R, the addition of 5-HT induced a concomitant reduction in mean neurite length (Fig. 4B, left), but this is not observed as a change specific to the primary neurite population (Fig. 4B, right).

No significant changes in the number of secondary neurites were observed with 5-HT stimulation in the absence or presence of 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R antagonists (Fig. 5A, left). Nonetheless, the addition of 5-HT promoted an increase in the number of long secondary neurites, although it was not statistically significant. Furthermore, the treatment with 100 nM 5-HT significantly increased the mean length of secondary neurites per

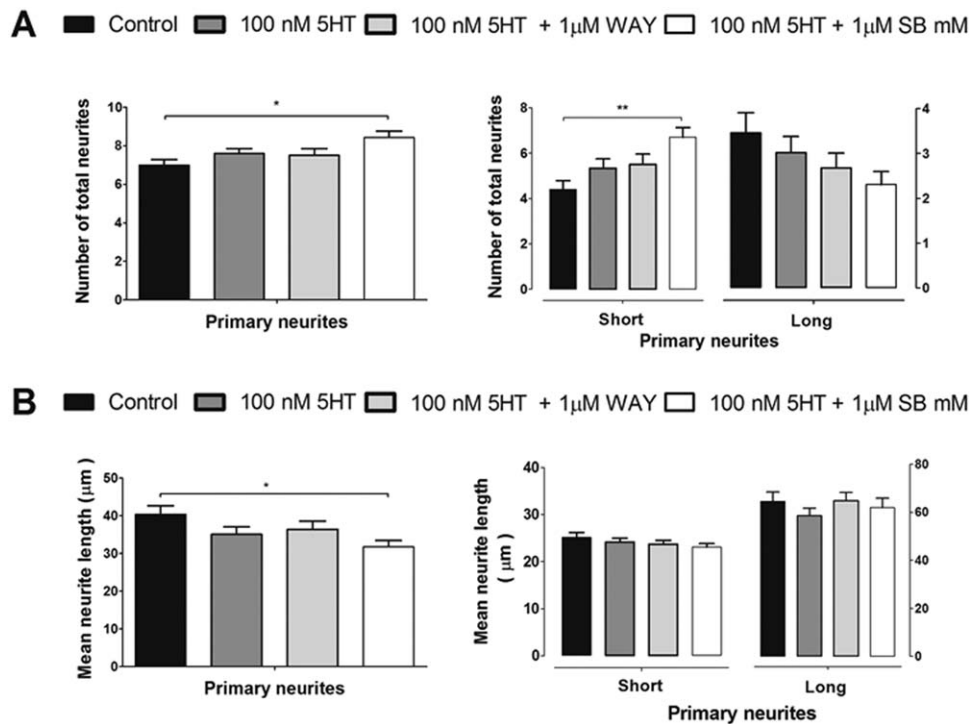


Fig. 4. Effect of 5-HT on the number and mean length of primary neurites. Cultures were incubated for 24 hr with 100 nM 5-HT. The antagonists WAY-100635 and SB269970 were added 30 min prior to 5-HT. Number (A) and mean length (B) of primary neurites were determined for each individual hippocampal neuron in NeuroJ soft-

ware. Neurites classified as primary were segregated according to their length and designated as short ( $\leq 40 \mu\text{m}$ ) or long ( $>40 \mu\text{m}$ ) primary neurites. The data represent mean  $\pm$  SEM. Data were analyzed by ANOVA for each condition followed by the Bonferroni post hoc test. \* $P < 0.05$ , \*\* $P < 0.01$ .

neuron from  $13.1 \mu\text{m}$  ( $\pm 1.4$ ) to  $19.2 \mu\text{m}$  ( $\pm 2.2$ ; Fig. 5B, left). In contrast to the slight effect of  $1 \mu\text{M}$  WAY-100635, the treatment with  $1 \mu\text{M}$  SB269970 inhibited the 5-HT-induced outgrowth of secondary neurites. To determine whether this inhibition represented a heterogeneous response, we evaluated the effect of 5-HT and receptor antagonist on secondary neurites segregated according to their length. As shown in Figure 5B (right), WAY-100635 prevented the 5-HT-induced increase in the mean length of short secondary neurites. Similarly, SB269970 prevented the 5-HT-induced increase in the mean length of short secondary neurites (Fig. 5B, right). 5-HT did not, however, increase the mean length of long secondary neurites (Fig. 5B, right).

## DISCUSSION

Several studies support the idea that neurotransmitter release during development of the central nervous system is critical for proper brain development (van Kesteren and Spencer, 2003). In addition to acting as a neurotransmitter, 5-HT may modulate several processes at early stages of neuronal development (van Kesteren and Spencer, 2003). Pharmacological approaches have shown that 5-HT participates in cell differentiation, synaptogenesis, and axonal pathfinding in addition to its effect on cell migration and morphogenesis in the central nervous system

(Buznikov et al., 2001; Gaspar et al., 2003). Our data show that 5-HT stimulates secondary neurite outgrowth in cultured embryonic neurons from rat hippocampi. Inhibition by the selective antagonists WAY-100635 and SB269970 suggests that 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R may mediate 5-HT-induced neurite outgrowth.

## Distribution of 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R in the Brain During Development

Several studies show that 5-HT<sub>R</sub> subtypes are expressed early in embryonic development and are dynamically regulated during pre- and postnatal development (Dutton and Barnes, 2008). Hippocampal neurons begin to express 5-HT<sub>1A</sub>R at about E16, just 1–2 days after completing mitosis and before migrating to the laminar layer (Patel and Zhou, 2005). Additionally, Patel and Zhou (2005) have demonstrated that the onset of 5-HT<sub>1A</sub>R expression occurs prior to serotonergic innervation (E19). During postnatal development, 5-HT<sub>1A</sub>R is initially detected in the soma and is sparsely distributed among the basal and apical dendrites and spines (Patel and Zhou, 2005). This distribution pattern is observed in both pyramidal and granular neurons of the hippocampus, indicating that there is a modification in receptor targeting as the neurons mature (Patel and Zhou, 2005). Additionally,



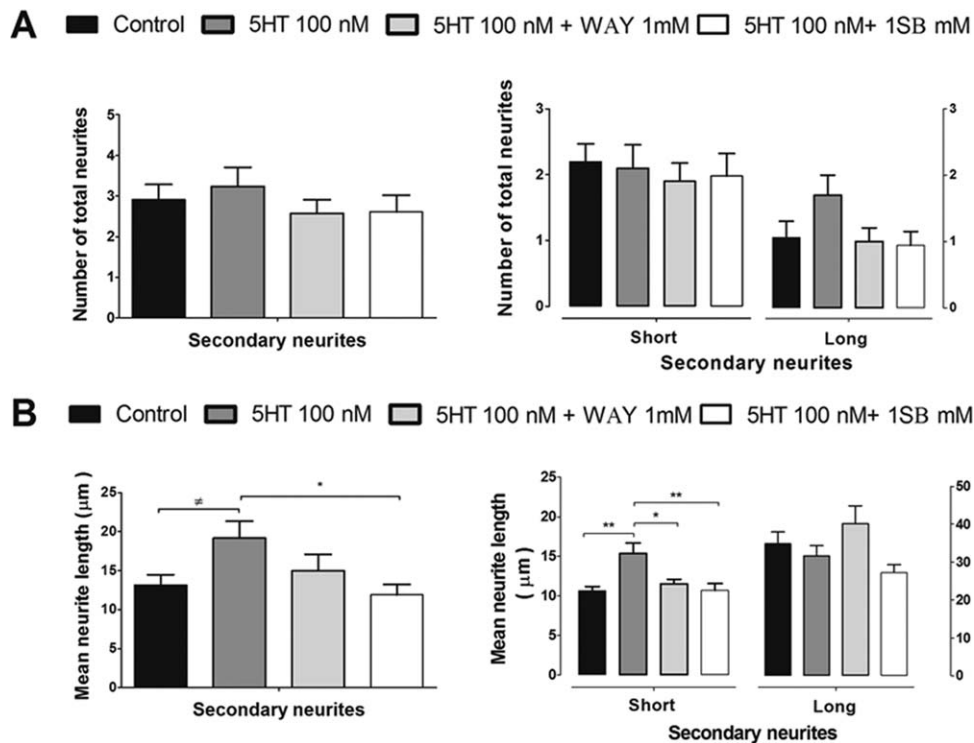


Fig. 5. Effect of 5-HT on the number and mean length of secondary neurites. Cultures were incubated for 24 hr with 100 nM 5-HT. The antagonists WAY-100635 and SB269970 were added 30 min prior to 5-HT. Number (A) and mean length (B) of secondary neurites were determined for each individual hippocampal neuron in NeuronJ. Neurites classified as secondary were segregated according to their

length and designated as short ( $\leq 20 \mu\text{m}$ ) or long ( $> 20 \mu\text{m}$ ) secondary neurites. The data represent mean  $\pm$  SEM. The data were analyzed by two-sided *t*-test for comparison of control vs. 5-HT,  $^{\#}P < 0.05$  (B, left). The effect of treatments on each group of secondary neurites was analyzed by ANOVA followed by the Bonferroni post hoc test.  $^*P < 0.05$ ,  $^{**}P < 0.01$ .

levels of 5-HT<sub>7</sub>R mRNA are high from P2 to P6, before being substantially reduced later in development (Kobe et al., 2012). Variations in the levels of 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R during early stages may be related to their specific functions in hippocampal neuron development during the first phase of brain development.

We described the presence of mRNAs for 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R in neuronal cultures at 2 and 3 DIV. Additionally, immunoreactive bands for both 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R appear in a Western blot of protein extracts obtained from a primary culture of E18 rat hippocampal neurons. These results are consistent with the expression of 5-HT<sub>1A</sub>R in E16–20 neurons of Ammon's horn and in interneurons present in the dentate gyrus, stratum radiatum, and stratum oriens of the hippocampus (Patel and Zhou 2005). Furthermore, we detected two isoforms of 5-HT<sub>7</sub>R (5-HT<sub>7a</sub>R and 5-HT<sub>7b</sub>R) that are generated by alternative splicing (Heidmann et al., 1997). These receptor isoforms differ in their carboxyl terminal portion (Heidmann et al. 1997). Additionally, we observed that the ratio of these isoforms did not change from 2 to 3 DIV. These results demonstrate for the first time the presence of 5-HT<sub>7</sub>R in primary cultured neurons of E18 rat hippocampus.

## 5-HTRs and Neuronal Morphology

Only a few studies have evaluated the contribution of 5-HTRs to neuronal morphology. For example, Hayaishi et al. (2010) described the promotion of dendrite and axon formation in cortical GABAergic interneurons by the ligand-gated ion channel 5-HT<sub>3</sub>R. The expression of 5-HT<sub>4a</sub>R in mammalian cells promotes different effects through the activation of G $\alpha_{13}$  and RhoA (Ponimaskin et al., 2002), important regulators of the cytoskeleton (Strathmann and Simon, 1991; Buhl et al., 1995; Arber et al., 1998; Katoh et al., 1998; Yang et al., 1998). In addition, the expression of 5-HT<sub>4a</sub>R in neuroblastoma  $\times$  glioma hybrid NIE-115 cells promotes RhoA-dependent neurite retraction and cell rounding (Ponimaskin et al., 2002). Selective stimulation of endogenous 5-HT<sub>4</sub>R in mouse hippocampal neurons by the agonist BIMU8 promotes a decrease in the length and number of neurites (Kvachnina et al., 2005). This observation suggests a functional role for 5-HT<sub>4</sub>R during development.

This study used rat hippocampal neurons and stimulation with 5-HT at 2 DIV for 24 hr. Sholl analyses show that 5-HT had no significant effect on neuron complexity (branching points and end points). After 5HT<sub>1A</sub>R was blocked, however, the addition of 5-HT for 24 hr slightly



increased the number of intersections (Fig. 2) within a 10- $\mu$ m radius from the soma. Additionally, after 5-HT<sub>7</sub>R was blocked, the addition of 5-HT promoted a significant increase in the number of short primary neurites, with a concomitant reduction in the number of long primary neurites, though not significant. This observation suggests that the activation of 5-HT<sub>7</sub>R could inhibit neuritogenesis and probably favors the growth of long neurites. In future experiments, it will be important to determine whether 5-HT<sub>1A</sub>R and 5HT<sub>7</sub>R influence the neuritogenesis process as early as 1 DIV.

In the present study, we observed that 5-HT increases the mean length of secondary neurites, specifically of those that are short ( $\leq 20$   $\mu$ m in length). This result can be interpreted as an effect on outgrowth of neurites that are more sensitive to growth cues, which suggests a specific spatial role of 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R in neuron growth. Further research could be conducted to determine whether 5-HT can promote primary neurite outgrowth at an early stage of culture and modify axon definition. By using specific antagonists, we showed that both 5-HT<sub>7</sub>R and 5-HT<sub>1A</sub>R may be involved in neuron growth. Stimulation with 5-CT, a 5-HT<sub>7</sub>R agonist, has been shown to increase the length of total neurites in hippocampal cultures obtained from P1–2 mice (Kvachnina et al., 2005). This effect may be similar to that observed in our results; however, Kvachnina et al. evaluated neither the presence of 5-HT<sub>7</sub>R nor the differences between primary and secondary neurites, as we did. Additionally, they stimulated 5-HT<sub>7</sub>R with 5-CT after 4 hr of plating, a neuronal stage at which cell polarity has not been established (Kvachnina et al., 2005). Another variable in the effect of 5-HT<sub>7</sub>R on neuronal morphology is the duration of agonist stimulation. For example, it has been shown that primary cultured hippocampal neurons from E16 mouse embryos, when treated for 72 hr with 100 nM of the selective 5-HT<sub>7</sub>R agonist AS19, show significant increases in total and mean neurite length and in neurite number (Tajiri et al., 2012). In contrast to those studies, we evaluated the effect of 5HT on neurons at 2 DIV over a period of 24 hr.

Additionally, studies in Neuro2A cells transfected with the 5-HT<sub>1A</sub>R and SK-N-SH cells endogenously expressing 5-HT<sub>1A</sub>R showed that stimulation by the 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R agonist 8OH-DPAT increases the percentage of cells with neurites; this effect was prevented by WAY-100169, an antagonist of 5-HT<sub>1A</sub>R (Fricker et al., 2005). More recently, studies in which 5-HT<sub>1A</sub>R knockout mice were used demonstrated that activation of 5HT<sub>1A</sub>R with 5-HT decreases the number of branching points of tertiary dendrites in hippocampal neurons at 4–7 DIV (Ferreira et al., 2010).

More recently, Speranza et al. (2013) reported that the enhancement of neurite outgrowth in cortical and striatal primary cultures promoted by 5-HT<sub>7</sub>R is related to the activation of the ERK and Cdk5 pathways. Previously, Kvachnina et al. (2005) reported that 5-HT stimulation of NIH-3T3 cells expressing 5-HT<sub>7</sub>R increases the activities of RhoA and Cdc42, effects that are associated

with increased G $\alpha_{12}$  activity. Additionally, activation of Rac1 and Cdc42 in cultured neurons promotes neurite branching and extension, respectively, whereas RhoA activation causes neurite retraction (Govek et al., 2005). In addition to the effects of 5-HTRs, the effects of small GTPases on neuronal morphology in hippocampal primary cultures should be explored in future studies. Furthermore, because the observed morphological effects are global, including processes related to both growth and neurite retraction, it will be important to determine whether retraction or outgrowth is preferentially affected by the activation of specific receptors.

Collectively, these results suggest that at an early stage of neuronal development 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R may have similar morphological effects in hippocampal neurons. Although our study does not evaluate the mechanism involved in the morphological changes, it is plausible that the 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R signalling pathways converge at some point and produce the same final morphological effect of neurite outgrowth. Alternatively, one receptor may negatively regulate the other receptor through heterodimerization. Indeed studies have shown that 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R form heterodimers both in vitro and in vivo (Renner et al., 2012). Heterodimerization decreases 5-HT<sub>1A</sub>R-mediated activation of G<sub>i</sub>-protein with no effect on 5-HT<sub>7</sub>R-mediated signalling (Renner et al., 2012). Renner et al. (2012) also showed that 5-HT<sub>7</sub>R expression is decreased in the hippocampus during development, which suggests that the proportion of heterodimers may change during development and thus alter the physiological contribution of each receptor to hippocampal development.

## CONCLUSIONS

The subtypes of serotonergic receptors are expressed early in embryonic life, and their expression and/or localization changes during pre- and postnatal development, suggesting a critical role during central nervous system development and maturity. The most important finding of the present study is that stimulation of endogenous 5-HT<sub>1A</sub>R or 5-HT<sub>7</sub>R produces morphological changes, inducing the outgrowth of shorter secondary neurites in hippocampal neurons. Therefore, our study adds new evidence supporting the involvement of 5-HT in neuronal morphology at an early stage of development through the participation of both 5-HT<sub>1A</sub>R and 5-HT<sub>7</sub>R. The transduction pathway involved in these effects, however, remains to be elucidated.

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