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Serotonin Depletion Hampers Survival and Proliferation in Neurospheres Derived from Adult Neural Stem Cells

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Serotonin (5-HT) and the serotonergic system have recently been indicated as modulators of adult hippocampal neurogenesis. In this study, we evaluated the role of 5-HT on the functional features in neurospheres derived from adult neural stem cells (ANSC). We cultured neurospheres derived from mouse hippocampus in serum-free medium containing epidermal (EGF) and type-2 fibroblast growth factor (FGF2). Under these conditions ANSC expressed both isoforms of tryptophane-hydroxylase (TPH) and produced 5-HT. Blocking TPH function by para-chlorophenylalanine (PCPA) reduced ANSC proliferation, which was rescued by exogenous 5-HT. 5-HT action on ANSC was mediated predominantly by the serotonin receptor subtype 5-HT1A and, to a lesser extent, through the 5-HT2C (receptor) subtype, as shown by selectively antagonizing these receptors. Finally, we documented a 5-HT-induced increase of ANSC migration activity. In summary, we demonstrated a powerful serotonergic impact on ANSC functional features, which was mainly mediated by 5-HT1A receptors.

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INTRODUCTION

Neuroplasticity enables the brain to receive information and to respond appropriately to endogenous and or exogenous stimuli. The presence of stem cell niches that sustain *de novo* generation of neurons in the adult brain represents one of the most fascinating facets of neuroplasticity. Two known germinal zones continue to generate new neurons and glia in the adult mammalian brain: the subventricular zone (SVZ), lining the lateral ventricle, and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus (Alvarez-Buylla *et al*, 2002; Gage, 2000; Ming and Song, 2005; Santarelli *et al*, 2003). Besides the SVZ and SGZ, a large number of proliferating cells are found between the hippocampus and the corpus callosum in the adult rodent brain, a region that has recently been named as the subcallosal zone (Seri *et al*, 2006). These regions contain stem cells that can be isolated and grown *in vitro* as neurospheres, maintaining self-renewal and multipotentiality over time (Gage *et al*, 1998; Gritti *et al*, 2002; Reynolds and Weiss, 1992).

A plethora of factors modulate adult neurogenesis by regulating neural stem cell proliferation, survival or fate choice. Mitogens such as epidermal growth factor (EGF), type-2 fibroblast growth factor (FGF2) (Gritti *et al*, 1995, 1996, 1999; Kuhn *et al*, 1997) and transforming growth factor alpha (TGF-α) (Tropepe *et al*, 1997; Weickert and Blum, 1995) induce adult neural stem cells (ANSC) proliferation. Ciliary neurotrophic factor, platelet-derived growth factor , bone morphogenetic protein 2 (Gross *et al*, 1996), brain-derived neurotrophic factor (Benoit *et al*, 2001; Li *et al*, 2008), and members of the wnt protein family (Lie *et al*, 2005) influence cellular fate selection and differentiation outcome in neural stem cell progeny (Jagasia *et al*, 2006). Moreover, many of these factors exert similar effects on ANSC both *in vivo* and *in vitro*.

This work is dedicated to Helmut Beckmann, the former chair of the Department of Psychiatry at the University of Wuerzburg

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The serotonergic system evolves early during development (Walther and Bader, 1999) and eventually represents one of the widest distributed neural networks in the adult brain. Chronic treatment with serotonergic antidepressants correlates with neurogenesis in the adult hippocampus. The production of new neurons may be required for the behavioral effects of these molecules—it may also indicate that progenitor survival, proliferation, and differentiation are modulated by serotonergic neurotransmission (Banasr *et al*, 2004; Brezun and Daszuta, 1999, 2000a, b; Jacobs *et al*, 2000; Santarelli *et al*, 2003).

Recently, a class of hippocampal stem cells has been indicated as the target of antidepressants (Encinas *et al*, 2006). However, the molecular mechanisms and pathways involved are not well understood meaning that a potential role of serotonin on other hippocampal progenitors or stem cells should be examined carefully.

To study the mechanisms by which the serotonergic system may impact on their functional features, we applied a standardized *in vitro* assay (NeuroSphere Assay, NSA) (Gritti *et al*, 1996, 1999; Reynolds and Rietze, 2005; Reynolds and Weiss, 1992; Seri *et al*, 2006) to isolate and expand ANSC grown in neurospheres from the mouse hippocampus. We showed that inhibition of 5-HT synthesis by TPH inactivation caused a dramatic fall in ANSC proliferation, which could be rescued by adding exogenous 5-HT. Moreover, we could show a novel effect of 5-HT on ANSC migration activity. On the level of 5-HT receptors, antagonist experiments showed that 5-HT1A and, to a lesser extent, 5-HT2C receptor subtypes mediated 5-HT action on ANSC.

Our work indicates ANSC as a reliable *in vitro* system to study the impact that neurotransmitters and drugs might have on neurogenesis and provides empirical support for their potential relevance in the development of therapeutic approaches to treat neuropsychiatric disorders (Dranovsky and Hen, 2006; Kempermann and Kronenberg, 2003; Warner-Schmidt and Duman, 2006).

MATERIALS AND METHODS

Establishment of Primary ANSC Cultures

Animals were treated according to NIH equivalent animal care rules. Adult wild-type C57B6 mice (3–6 months old) were anesthetized by intraperitoneal injection of pentobarbital (120 mg/kg) and killed by cervical dislocation. Brains were removed and placed in chilled PBS. Whole hippocampi were carefully removed and put in a digestion solution (EBSS containing 0.94 mg/ml papain (Worthington Biochemicals)), 0.2 mg/ml cystein and EDTA (both from Sigma) for 50 min at 37°C under gentle rocking. After digestion, tissues were washed twice in DMEM (Gibco Life), mechanically dissociated using a fire-polished Pasteur pipette, and finally placed in serum-free DMEM/F12 (1 : 1 v/v; Gibco Life) containing 20 ng/ml EGF and 10 ng/ml FGF-2 (both human recombinant; Peprotech), 2 mM L-glutamine, 0.6% glucose, 9.6 μg/ml putrescine, 6.3 μg/ml progesterone, 5.2 ng/ml sodium selenite, 0.025 mg/ml insulin, 0.1 mg/ml transferrin and 0.2 μg/ml heparin (all Sigma) (growth medium) at a density of 20 000 cells/ml onto sterile, non-coated Petri dishes (Corning).

Cell Culturing and Propagation

Cells were serially subcultured by mechanical dissociation every 4–7 days. Cells were collected as neurospheres and the total number of viable cells was assessed during each passage by trypan blue exclusion (Sigma). Self-renewal and multipotency were assessed at every three subculturing passages as previously described (Gritti *et al*, 2002) as proof of the 'stem-cellness' or multipotency of the ANSC.

For the experiments, ANSC were collected 5 days after the last subculturing passage. In some experiments ANSC were harvested 2 days after the last subculturing passage to obtain a cell fraction enriched in stem cells. ANSC at subculturing passages 3, 7, and 11 were used, obtaining reproducible results.

Cell Culture Treatments with Different Agents

ANSC were treated with different anti-serotonergic agents. In preliminary experiments, the appropriate concentration of each agent was determined to exclude cytotoxic effects, which could have been mistaken as effects on survival or proliferation. For inactivating 5-HT production, ANSC were treated with 50 μM para-chlorophenylalanine (PCPA, Sigma). For rescue experiments 5 μM 5-HT was used. The highly specific 5-HT1A antagonist *N*-(2-(4(2-methoxyphenyl)-1-piperazinyl-1-piperazinyl)-*N*-2-pyridinyl) cyclohexancarbonate (WAY100635; kindly provided by Dennis Murphy, NIMH, NIH, Bethesda, MD) and the 5-HT 2B/2C receptor antagonist SB 206553—N3-pyridinyl-3,5-dihydro-5-benzol-carboxamide-hydrochloride (Sigma) were applied at a concentration of 50 μM. PCPA was dissolved in 100% ethanol and prepared as a 1000 × stock solution, which was further diluted in culture media. All the other compounds were soluble in culture media and were freshly prepared for each experiment. Controls were treated with the same concentration of ethanol when PCPA was involved. Cells were counted by a hemocytometer in a blinded way without knowledge of treatments or status. Primary mouse skin fibroblast cell cultures grown in DMEM + 10% FCS were used as negative control.

Differentiation of Stem Cell Progeny, Immunocytochemistry, FACS Analysis

ANSC differentiation experiments and immunofluorescence assays were performed as described previously (Gritti *et al*, 1996). Briefly, undifferentiated ANSC were plated onto Matrigel-coated glass coverslips (Gibco Life) in growth medium. Three hours after plating cultures were either fixed (4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 20 min) and processed for the detection of TPH, or shifted to FGF2-containing medium, grown for 2 days, shifted to mitogen-free medium containing 2% FCS, grown for 4 days and then fixed and processed for immunofluorescence assays. In these cell cultures we evaluated the proportions of different neural cell types (neurons, astrocytes, and oligodendrocytes). Primary antibodies were: polyclonal sheep anti-TPH (1 : 100, AB1541, Chemicon), rabbit anti-GFAP (1 : 300, Chemicon), monoclonal anti-TUJ1 (1 : 1000, Covance), anti-GalCer (1 : 100, Chemicon), polyclonal rabbit anti-TPH1 and rabbit anti-TPH2 (1 : 400;

both kind gifts from Donald M Kuhn, Department of Psychiatry, Wayne State University (Sakowski *et al*, 2006)).

Primary antibodies were incubated for 2 h at room temperature before adding Texas-red conjugated donkey-anti-sheep (1 : 500), goat anti-mouse or goat anti-rabbit Alexa-488 or Alexa 546 (1 : 2000)-conjugated secondary antibodies (Chemicon) for 30 min at room temperature. Coverslips were counterstained with DAPI (1 : 1000 dilution of stock solution, 10 min at RT). Samples were examined and photographed using a Nikon Eclipse 3000 fluorescence microscope at 640-fold magnification. Immunoreactive cells were counted in at least five non-overlapping fields in each sample (>500 cells/sample) and expressed as a percentage of the total number of nuclei. Confocal images were taken with a Leica TCS SP2 three-laser confocal microscope. Fluorescent signals from single optical sections were sequentially acquired and analyzed by Photoshop 7.0 (Adobe). For fluorescence-activated cell sorting (FACS), anti-TPH antibody (0.2 μg/ml), and PE-conjugated secondary antibody (Chemicon) were used. Samples were analyzed using a FACSCalibur (Beckton and Dickinson).

Determination of Cell Proliferation

ANSC (30 000 cells/cm^2) were plated on 10 mm Matrigel-coated glass coverslips in growth medium containing 1 μM BrdU (Roche) for up to 48 h. During this period all the cells entering S-phase incorporated the thymidine analog. Cells were fixed in 4% paraformaldehyde and stained with a monoclonal anti-BrdU antibody (ready to use; Amersham). Nuclei were labeled with DAPI. Roughly one thousand cells (identified by DAPI staining) were counted per coverslip. The labeling index (LI) was calculated as number of BrdU-labeled cells/total number of nuclei and used as an index for proliferation. Experiments were performed in triplicate with comparable results.

Determination of Cell Number and Viability by MTT Assay

Cells were plated onto Matrigel-coated 96-well plates (10 000 cells/well). At every time point, one hour before harvesting, the tetrazolium dye 3-(4,5-dimethylthiazol-2)-2,5-diphenyl-2H-tetrazolium bromide (MTT; 5 mg/ml in PBS; Sigma) was added to the medium (final dilution: 500 μg/ml). The pale yellow redox indicator MTT is reduced to a dark blue product, MTT-formazan, by the mitochondrial dehydrogenases of living cells. Following 1-h incubation at 37°C, the medium was discarded and cells were lyzed by adding 50 μl of DMSO. After 15 min at room temperature, MTT reduction was measured spectrophotometrically at 550 nm wavelength.

Migration Assay

For cell migration experiments cells were assayed in blind well or Boyden chambers (New Technologies Group-NTG). In brief, PVP-free polycarbonate filters with 8 μm pores (Costar) were coated with Matrigel over night. DMEM medium (negative control), DMEM plus 5 μM serotonin or growth medium (positive control) were placed in the lower chambers. ANSC were used for the chemotaxis assays 24 h after the last subculturing passage. Cells (50 000) resuspended in 200 μl DMEM were placed in the upper chambers and incubated at 37°C in 5% CO_2 for 6 h. ANSC remaining on the upper surface of the filters were mechanically removed, whereas those migrated to the lower surface were fixed with ethanol, Giemsa stained, and counted at 400 × in 5 random fields per filter. Assays were performed in duplicate and repeated four times in independent experiments.

Reverse Transcriptase-PCR (RT-PCR)

Total RNA from ANSC was extracted using the RNeasy Mini kit (Qiagen). CDNA was obtained using Superscript RNase H-reverse transcriptase (Gibco Life). For analysis of gene expression, the following RT-PCR primer pairs were used: TPH: 5′-GTGGCTATCGGGAAGACAAC-3′ and 5′-AGACATCCTGGAAGCTCGTG-3′; TPH2: 5′-TGATGTTTCCAGTAAATACTGGG-3′ and 5′-CTCAGAGATCTTCCGAGGGAAC-3′; 5-HT 1A: 5′-CGCGCTA-GACAGGTACTGG-3′ and 5′-CGGGGACATAGGAGGTAGC-3′; 5-HT 2C 5′-GCCTATTGGTTTGGCACTT-3′ and 5′-CCATAATTGTCAACGGGATG-3′, 5-HT 2B 5′-GTGGGAACATCCTTGTGATTCT-3′ and 5′-GACCCAAAGACCATAAAACTGC-3′; TH 5′-AATTCCCCACGTGGAATACA-3′ and 5′-GTGCAGGAGCTCTCCATAGG-3′ and the house keeping gene GAPDH for control and semi-quantitative analysis. RT-PCR amplification consisted of 38 cycles with primer annealing at 60°C (TPH2, 5-HT2B and 2C receptor), 58°C (TPH1 and TH), and 54°C (5-HT1A receptor), respectively.

Semi-Quantitative PCR (Q-PCR)

Total RNA was isolated from the ANSC using Trizol Reagent (Life Technologies). First strand cDNA was then synthesized from 1 μg of total RNA with oligo $(dT)_{18}$-primers using the Promega RT-PCR System (Promega). Semi-quantitative real-time PCR was performed using the Mx3000 Real-Time PCR System from Stratagene (Stratagene) in the presence of SYBR Green. Each PCR (25 μl total volume) contained 1 μl of cDNA template, 12.5 μl of SYBR Green Jumpstart Taq ReadyMix for QPCR (Sigma) and 10 μM forward and reverse primers. Primers were designed by the PCR designer tool (Stratagene: labtools.stratagene.com) and synthesized by Primm (Milan, Italy). Primer sequences were as follows: TPH1: 5′-GCAAACAAGATGGACAGC-3′ and 5′-TTACAGGCAATCTTGGGA-3′; TPH2: 5′-CAGGAGAGGGTTGTCCTT-3′ and 5′-ACTGCTGTCTTGCTGCTC-3′; POLR2a: 5′-TCTCATCGAGGGTCATACCA-3′ and 5′-TCTGACGCAATGTGTTTCCT-3′. The thermal conditions were as follows: 1 cycle, 10 min at 95°C; 40 cycles of: 30 s at 95°C, 1 min at 60°C and 1 min at 72°C. To confirm amplification specificity the resulting PCR products were subjected to a melting curve analysis and subsequent agarose gel electrophoresis. Total RNA extracted from E18 mouse embryo was used as positive control, whereas PCR runs without cDNA served as negative controls. RNA polymerase 2 subunit A (PolR2a) was used to normalize each template, because PolR2a has been shown to produce more accurate normalization than comparable housekeeping genes (Saviozzi *et al*, 2006; Wacker and Godard, 2005).

All these experiments were done in duplicates and repeated three times with similar results.

Determination of 5-HT and 5-Hydroxyindolacetic acid (5-HIAA) by HPLC

Supernatants derived from 5-day-old serially passaged neurospheres were used. Culture media kept for 5 days in the same incubator in the absence of cells was used as negative control. Samples were sonicated under argon in ice-cold H_3PO_4 (150 mM) and diethylenetriamine pentaacetic acid (500 μM), centrifuged at 35 000 *g* for 20 min at 4°C, as described previously (Vogel *et al*, 2003). Briefly, the supernatant was filtered through Millipore (Bedford) Ultrafree-MC filter cups at 9000 *g* for 1–2 h at 4°C. For the analysis of 5-HT and 5-hydroxyindolacetic acid (5-HIAA), 50 μl portions of the supernatants were injected directly into an HPLC system with electrochemical detection at +0.75 V using EC 250/4 Nucleosil 120-5-C18 reversed-phase chromatography columns (Machery-Nagel) with mobile phase consisting of 84% 0.1 M NaH_2PO_4, pH 3.35, 16% MeOH, 0.65 mM octanesulfonic acid, 0.50 mM triethylamine and 0.1 mM EDTA.

Statistical Analysis

Experimental data are presented as mean ± SD. Statistical significance was defined at the $p < 0.05$ level. Growth curve and MTT assay data were analyzed statistically by ANOVA following Student's *t*-test when two groups were involved.

RESULTS

The main purpose of this investigation was to study the impact of the serotonergic system on proliferation and survival of adult neural stem cells derived from mouse hippocampus. In our *in vitro* model, hippocampus-derived ANSC were isolated and expanded as neurospheres. This neurosphere assay (NSA) (Reynolds and Rietze, 2005) relies on cells exposed to mitogens such as EGF and FGF2, which had been plated after dissociation of brain tissue. Under these selective culture conditions, only NSCs and highly undifferentiated progenitors proliferate and form clonal aggregates called primary neurospheres, whereas committed precursors and terminally differentiated cells are progressively eliminated from the culture. Upon subculturing, primary neurospheres can generate secondary neurospheres, representing the renewal of the previous population. This method allows the establishment of long-term expanding NSC lines that maintain stable proliferation and multipotency (the ability to give rise to astrocytes, oligodendrocytes, and neurons) over time.

ANSC Express Tryptophan Hydroxylase 1 and 2 and Produce 5-HT

We first sought to prove TPH protein expression by immunostaining (Figures 1a and 2c) and FACS analysis (Figure 1c). The large majority (94 ± 0.5%) of undifferentiated ANSC maintained in growth medium displayed

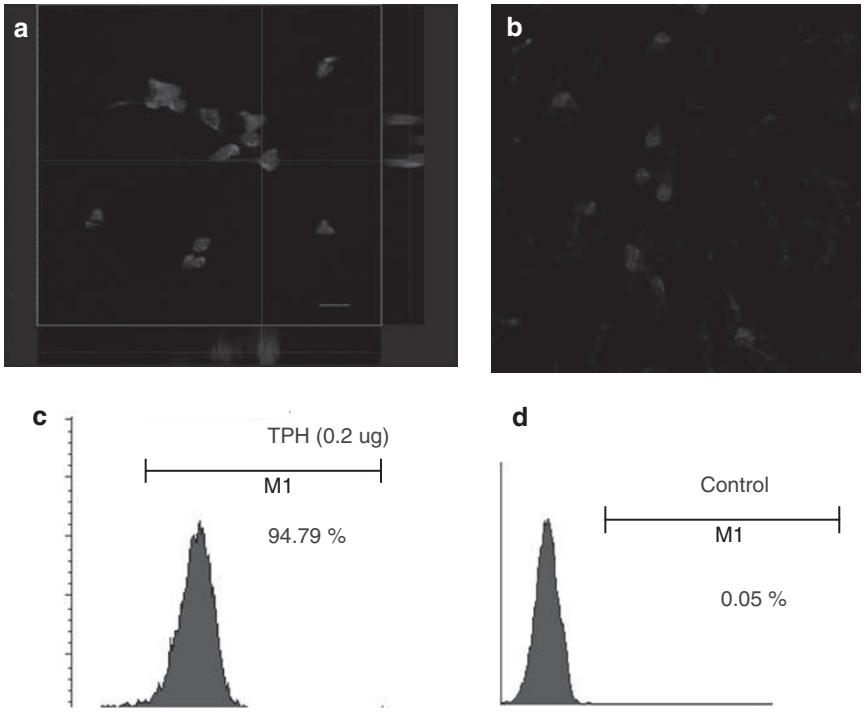

Figure 1 Presence of both TPH1 and TPH2 in ANSC. (a) Reverse transcriptase PCR reveals the presence of mRNA for both TPH1 (upper picture, lane 3, 487 bp) and TPH2 (lower picture, lane 3, 451 bp) in undifferentiated ANSC (lane 1-total mouse brain, positive control; lane 2, mock probe-negative control). (b) Quantitative PCR reveals a 1 : 1.2 ratio (normalized to GAPDH expression levels) of TPH1 to TPH2 mRNA expression in undifferentiated ANSC ($p < 0.05$). (c) Immunofluorescence of TPH1 and TPH2 protein isoforms (red) confirming PCR (both isoform-specific polyclonal rabbit anti-mouse antibodies used at 1 : 400 dilution, DAPI counterstain describing nuclei; (d) negative control by omitting the primary antibody). TPH1 and TPH2 visualized by Zeiss Axiovert fluorescence microscope, scale bar representing 10 μm.

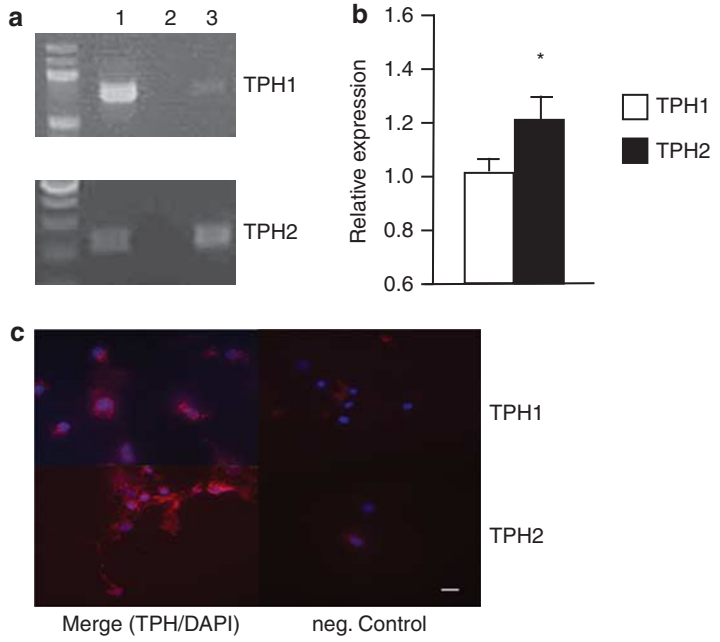

Figure 2 Quantization and expression of TPH protein in ANSC. (a) Pan TPH immunoreactivity in undifferentiated ANSC. The protein (red) was present in the cytoplasm, following a well-described expression pattern, as shown by confocal imaging (*z* axis stacks) scale bar −20 μm. (b) Negative control (no primary antibody). (c) Analysis of TPH immunoreactivity by FACS confirmed that more than 90% of ANSC express TPH. Control samples consisted of ANSC labeled with PE-conjugated secondary antibody (no primary antibody).

TPH immunoreactivity by immunofluorescence (Figure 1a) and FACS analysis (Figure 1c), whereas low numbers of TPH-expressing cells (2.4 ± 0.5%) were detected in adult murine skin fibroblast cultures (data not shown). No immunoreactive cells were observed in cultures when the primary antibody was omitted (Figure 1b and d). The antibody used for FACS recognizes both isoforms. Additionally, to investigate if a specific isoform predominates, we looked into TPH1 and TPH2 expression in serially passaged (passage 3–11) ANSC by reverse transcriptase (RT) PCR. Both TPH1 and TPH2 mRNA isoforms were detected in ANSC (Figure 2a) and expression levels did not change over time (data not shown). Quantitative PCR revealed a 1 : 1.2 ratio (normalized to PolR2A expression levels) of TPH1 compared with TPH2 mRNA expression in undifferentiated ANSC (Figure 2b). Finally, by using polyclonal rabbit anti-mouse antibodies specific for each TPH isoform (Sakowski *et al*, 2006) we confirmed PCR results, showing that both TPH1 and TPH2 proteins are present in undifferentiated ANSC (Figure 2c).

The presence of 5-HT and its metabolite 5HIAA in supernatants of cultured ANSC was confirmed by means of HPLC analysis (Figure 3).

Inactivation of 5-HT Impedes Proliferation of ANSC

We subsequently investigated the potential role of 5-HT on ANSC (proliferation) by suppressing TPH activity in ANSC maintained in growth media by using PCPA, a selective inhibitor known to drastically reduce brain 5-HT levels (Koe, 1971; Richard *et al*, 1990). In our experiments, a 3-day treatment with PCPA caused a significant drop in ANSC proliferation, as detected by BrdU incorporation and determination of the labeling index (LI) (LI = 17 ± 10 and 44 ± 18% in PCPA-treated and PCPA-untreated control cells, respectively) (Figure 4a).

Similarly, the rescue assay revealed a significant decrease in PCPA treated with respect to untreated cells. After 3 and 7 days of PCPA treatment, ANSC were reduced to 60 and 40%, respectively (Figure 4b). If the decrease in ANSC proliferation were the result of a decreased endogenous 5-HT synthesis due to TPH inhibition, addition of exogenous 5-HT to the media should be able to stop or even reverse this process. Indeed, adding 5-HT after 3 days of PCPA treatment completely rescued proliferation (Figure 4b) of ANSC to control levels.

The Serotonergic Machinery in ANSC Mainly Functions via 5-HT1A Receptors

To identify the signaling pathway modulating ANSC proliferation we examined the different 5-HT receptor subtypes expression in ANSC by RT-PCR. Undifferentiated ANSC grown in culture medium containing EGF and FGF-2 expressed 5-HT1A and 2C (but not 5-HT2B) receptor mRNA (Figure 5a). For unification of the PCR results we then undertook functional assays. To further characterize the impact of the 5-HT1A receptor, we used WAY100635, a potent and selective antagonist (Gozlan *et al*, 1995). Treatment with WAY100635 (72 h, 50 μM) reduced ANSC viability (77.0 ± 4.3% of control), whereas the same concentration of SB206653 (inhibitor of 5-HT2C and 5-HT2B receptor) did not significantly reduce the cell number (96.3 ± 5.4% of control) (Figure 5b). Absence of 5-HT2B mRNA expression in ANSC indicates that SB206653 acts specifically on the 5-HT2C receptor, with no cross-reaction with the 5-HT2B receptor. A similar effect was observed on ANSC long-term proliferation, which was

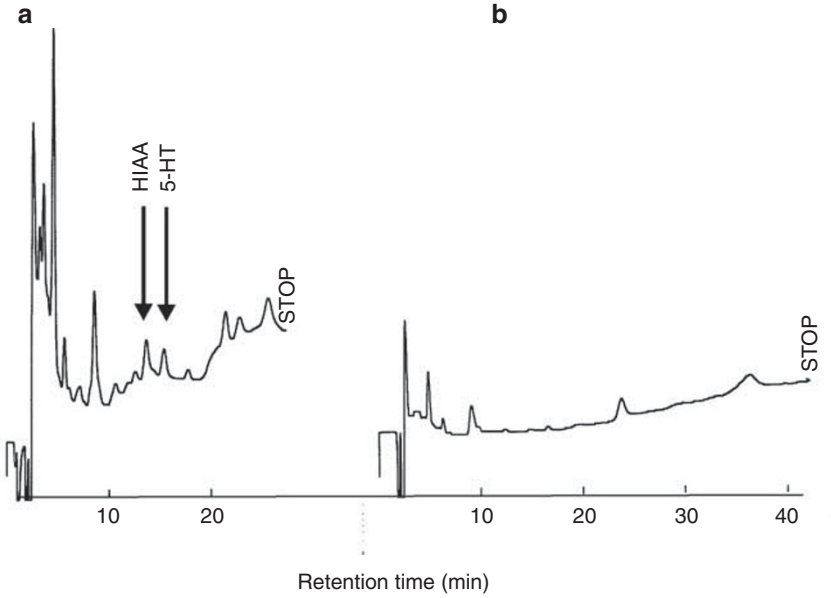

Figure 3 HPLC analysis of serotonin content in the culture media. (a) Culture media from ANSC (5 days of incubation) revealed presence of 5-HT and its metabolite 5-HIAA, whereas no 5-HT levels were detected in the control media (b).

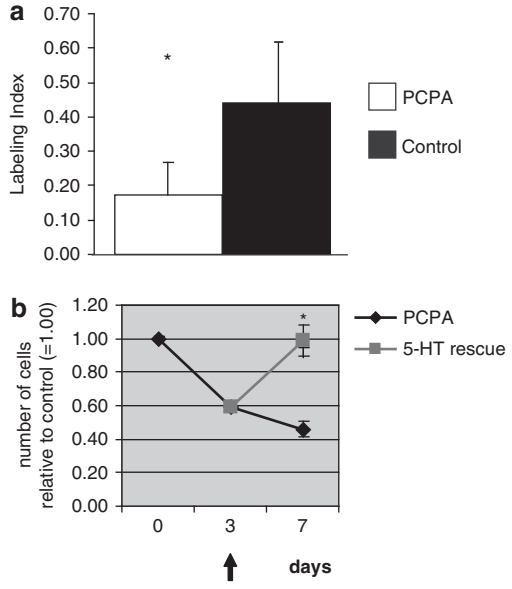

Figure 4 Exogenous 5-HT rescues PCPA-induced decrease of ANSC proliferation and survival. (a) When 50 μM PCPA was applied for 3 days, a significant ($p<0.05$) drop in proliferation (LI = 17 ± 10%) occurred compared with control cells (LI = 44 ± 18%). Labeling index indicates the amount of cells that incorporated BrdU on the total number of DAPI-stained nuclei. (b) Neurosphere assay shows that a 3- and 7-day treatment of ANSC with PCPA (50 μM) decreased the cell count to 40 and 60%, respectively, compared with control level (100%). Addition of 5-HT during the last 4 days of PCPA treatment is sufficient to rescue ANSC to control levels (*$p<0.05$).

reduced by blocking the 5-HT1A, but not the 5-HT2C receptor.

The outcome of these assays prompted us to introduce WAY100635 and SB206653 in the settings of 5-HT rescue experiments described above (Figure 4a). In this modified setting, the ANSC were treated with PCPA for 3 days and then 5-HT alone or 5-HT + WAY100635 or 5-HT + SB206653 were added for the remaining 4 days. These experiments showed that blocking 5-HT1A receptors (but not 5HT2C receptors) hampered 5-HT-induced rescue of ANSC ($p<0.001$), which returned to levels similar to PCPA-treated ANSC (Figure 6). This suggests that ANSC' response to exogenous 5-HT is mainly the result of 5-HT1A receptor subtype activation.

5-HT Promotes ANSC Migration

We further investigated the potential effect of 5-HT on another ANSC functional property, their migratory activity. Migration assays using Boyden chambers showed that exposure of ANSC to 5-HT (5 μM) for 6 h resulted in a 10-fold increase in the number of migrated cells with respect to ANSC exposed to growth factor and 5-HT-free medium (negative control). This level of migration activity was close to that observed in response to EGF and FGF2, two known growth factors promoting ANSC migration and considered as a positive control in our experimental setting (Figure 7).

DISCUSSION

This study proposes possible mechanisms by which the serotonergic system impacts on ANSC proliferation, suggesting an auto-regulatory pathway of serotonergic activity on ANSC in an *in vitro* neurosphere assay. The serotonergic machinery required for this seems to be minimal, mainly involving TPH (the decisive enzyme in 5-HT biosynthesis). In an autocrine and paracrine manner 5-HT stimulates proliferation and enhances survival of ANSC via the 5-HT1A receptor. There is apparently a marginal role, if any, for serotonin transporter (5-HTT)-driven fine-tuning of 5-HT in ANSC, contrary to the situation in differentiated neurons (Schmitt *et al*, 2007). This suggests that cellular and molecular pathways, through which 5-HT exerts its growth-

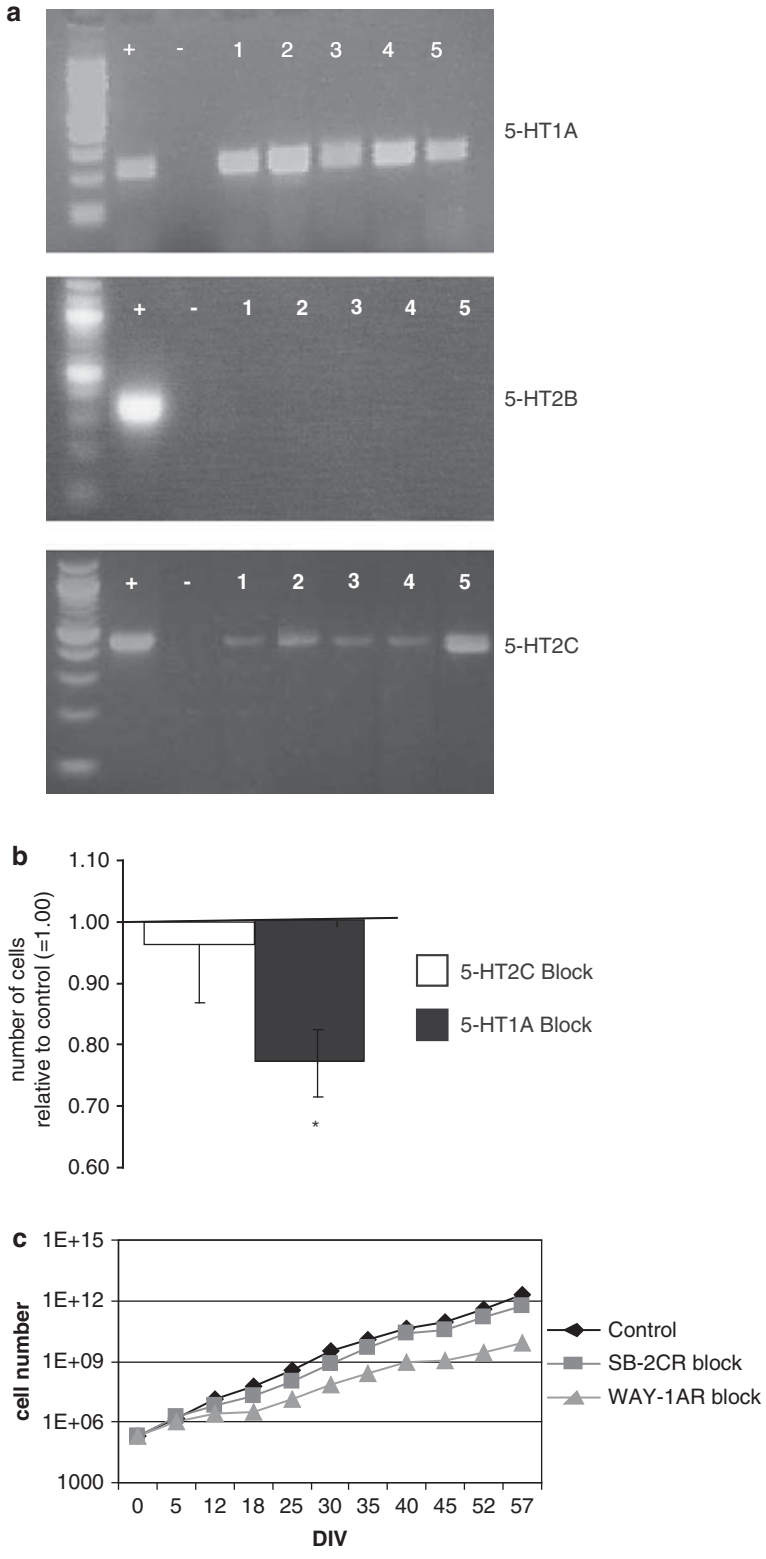

Figure 5 Functional assays reveal a major impact of the 5-HT1A receptor. (a) Reverse transcriptase PCR shows expression of 5-HT1A (556 bp) and 5-HT2C (645 bp) receptor mRNA, whereas 5-HT2B (456 bp) is not expressed. Lane 1: precursors, passage (P) 3; lane 2: precursors, P11; lane3: neurospheres P3; lane 4: neurospheres P11; lane 5: differentiated cells P11; + positive control: total mouse brain; −negative control: mock probe. (b) Neurosphere assay shows that 5-HT1A receptor blockade by WAY100635 (50 μM) significantly ($p < 0.05$) reduces ANSCs compared with blockade of 5-HT2C receptor by SB206553 (50 μM) (about 30 and 5% reduction in survival with respect to control, respectively). (c) Population analysis showed no differences in long-term proliferation and growth rate (57 days, 11 subculturing passages) between SB206553-treated ANSC and the untreated population by contrast to the cells treated by WAY100635, which revealed a decreased long-term growth rate.

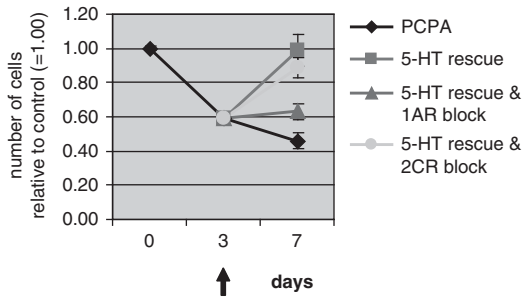

Figure 6 5-HT acts via the 5-HT1A receptor. WAY100635 (50 μM, red line), but non SB206553 (50 μM, yellow line) given in addition to 5-HT after 3 days of PCPA treatment (black line), partially inhibited 5-HT-induced rescue (green line), indicating the involvement of the 5-HT1A but not 5-HT2C receptor in mediating 5-HT action.

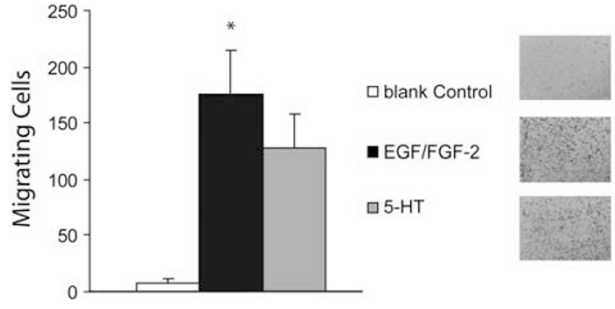

Figure 7 Serotonin induces ANSC migration. A 6-h incubation in growth factor-free medium in the presence of 5-HT (5 μM) increased the number of migrated ANSC (black column and lower panel) with respect to control cells (growth factor-free medium without 5-HT, empty column and upper panel), to levels close to those observed in response to EGF + FGF2 treatment (black column, middle panel), considered as our positive control (*$p < 0.05$). *Y* axis on the graph indicates the average number of migrating cells/field (10 fields/coverslip counted at ×40 magnification, $n = 5$ independent experiments).

factor-like properties, might be different from those necessary for its neurotransmitter function. Although there is converging evidence that the 5-HT1A receptor and 5-HTT modulate anxiety-related behavior, the potential function of 5-HTT in adult neurogenesis and the mechanisms engaging them in this process are complex and remain to be further characterized (Gardier *et al*, 2003; Schmitt *et al*, 2007).

A potential autocrine/paracrine serotonergic impact on ANSC implies the presence of a functional serotonergic machinery in these cells. Tryptophane-hydroxylase is the rate-limiting enzyme in serotonin synthesis. Until very recently serotonin was thought to be the gene product of one isoform, TPH is now known to exist in two isoforms: TPH1, mainly found in murine gut, pineal gland, spleen, and thymus; and TPH2, largely confined to brain structures (Gutknecht *et al*, 2009; Patel *et al*, 2004; Walther and Bader, 2003). Despite of this, the role of TPH1 in brain 5-HT synthesis is still poorly understood. TPH2 on the other hand has been shown to be expressed in brain areas besides the raphe nuclei, for example, inside the pituitary (Zill *et al*, 2009). Here, we showed that undifferentiated hippocampal ANSC express the transcripts and the proteins of both TPH isoforms by RT-PCR and immunohistochemical analysis using polyclonal antibodies known to retain specificity against each TPH isoform in immunoblotting, immunoprecipitation, and immunocytochemical applications (Sakowski *et al*, 2006). Although THP2 is predominantly expressed in mesencephalic serotonergic neurons, its presence has recently been described in brain extracts derived from the striatum as well as the hippocampus (Sakowski *et al*, 2006). In the absence of TPH2, there is still serotonergic input in the dentate gyrus and it is noteworthy that there is no change in raphe neuron formation and fiber distribution marking the particular role of TPH1 (Gutknecht *et al*, 2008). Further studies are needed to define the functional role of the two TPH isoforms in ANSC more precisely. Still, it should be emphasized that our data stated a so far unobserved co-expression of both TPH isoforms within the same cells, that is, ANSC.

Most importantly, our data showed that ANSC grown as neurospheres in a serum-free medium—thus excluding exogenous 'contamination'—produce and secrete 5-HT in the presence of selected mitogens as detected by HPLC analysis of ANSC supernatants. This led us to suggest a putative autocrine role of 5-HT as a growth or maintenance factor in ANSC. Survival and proliferation effects of 5-HT on neural progenitor cells have been shown *in vivo* (Brezun and Daszuta, 2000b) and its early presence in ES cells may be linked to a functional role as shown *in vitro* (Walther and Bader, 1999). Our data confirms this for ANSC. The 'trophic' activity of this neurotransmitter is also demonstrated in peripheral tissues, like in vascular smooth muscle cells (VSMC) where 5-HT remains a growth-modulating factor beyond the fetal period (Kawano *et al*, 2001). Recently, 5-HT has also been described as a survival factor of cardiomyocytes (Nebigil *et al*, 2003; Nebigil and Maroteaux, 2001).

Our results also indicate that 5-HT regulates survival, proliferation, and migration of ANSC. When 5-HT synthesis was abolished by PCPA, a significant decline in ANSC proliferation and survival was observed. All experiments were in line with *in vivo* reports showing that serotonergic deprivation by PCPA or the serotoninergic neurotoxin 5,7 dihydroxytryptamine (5,7-DHT) led to a significant reduction of dentate gyrus and SVZ neurogenesis in adult rats (Brezun and Daszuta, 1999; Walther and Bader, 1999). Our 5-HT rescue experiments confirmed the particular role of 5-HT as a maintenance factor in ANSC, because ANSC survival was rescued to levels of PCPA-untreated cells. A similar rescue effect was reported after grafting fetal raphe neurons into the 5,7-DHT lesioned hippocampus (Brezun and Daszuta, 2000b). This adds to a list of studies that are revealing unexpected effects of neurotransmitters on neural stem/progenitor cell function. Apart from the work on the glutamatergic NMDA receptor subtype (Cameron *et al*, 1995; Gould and Cameron, 1997; Nacher and Mcewen, 2006), the dopaminergic impact on stem/progenitor cell proliferation during brain development and in adult neurogenesis has also been examined (Borta and Hoglinger, 2007; Hoglinger *et al*, 2004; Kippin *et al*, 2005).

Serotonin acts through multiple receptors (Barnes and Sharp, 1999). As mentioned above, previous *in vivo* and *in vitro* studies showed the correlation between the serotonergic system and the neurogenic process (Brezun and Daszuta, 1999, 2000a, b). In this system, 5-HT1A receptors are of particular interest: in adult neurogenesis *in vivo* data

suggest that they can modulate cell proliferation in the hippocampus by a direct post-synaptic effect (Huang and Herbert, 2005). It is further known that antidepressant drugs drive neurogenesis in the hippocampus acting on the serotonergic system through 5-HT1A receptors (Gould, 1999; Malberg *et al*, 2000; Santarelli *et al*, 2003), whereas 5-HT1A antagonists decrease cell proliferation in the dentate gyrus (Radley and Jacobs, 2002). In addition, *in vitro* data showed the enhancement of EGF signaling and increased cell turnover rate in fibroblasts transfected with 5-HT1A receptor (Varrault *et al*, 1992a, b). We show here that ANSC express 5-HT1A and 5-HT2C receptors. In our experimental culture conditions ANSC are isolated and grown in the presence of EGF and FGF2, two mitogens that act synergistically to regulate ANSC proliferation and self-renewal (Gritti *et al*, 1995). Our functional data show that 5-HT rescue of PCPA-treated ANSC viability and proliferation was mainly mediated by 5-HT1A receptors. In terms of differentiation, we did not see any alteration in the number of neurons or glial cells generated by 5-HT-treated ANSC with respect to untreated cultures. Preliminary data indicate that TPH, 5HT, and 5-HT receptor levels are not consistently altered in differentiated cells with respect to the undifferentiated counterpart (data not shown).

These observations suggest that the 5-HT/5-HT1A system mainly acts on precursor/progenitor cells and is in line with a recent *in vivo* study in which the role of 5-HT1A and 5-HT2C antagonists in modulating neurogenesis was assessed (Banasr *et al*, 2004). Further studies on the 5-HT1A receptor subtype may also prove promising in terms of new pharmacologic treatment strategies (Blier and Ward, 2003; Lesch and Mossner, 1999). Inside the cells, we hypothesize a G_0/G_i-coupled pathway acting on cAMP as a conveyor of 5-HT1A activation (Hamon *et al*, 1990). Our data show that 5-HT2C blocking produces a non-significant decrease of ANSC cell survival and proliferation, thus suggesting a minor role of these pathways in regulating ANSC maintenance. However, these results might depend on the presence of insulin in ANSC growth media, because it has been described that insulin signaling can selectively inhibit 5-HT2C receptor activity (Hurley *et al*, 2003).

Importantly, levels of gene expression for these receptors are stable in ANSC at different subculturing passages, at least between passages 3 and 11 (roughly 3 months in culture). This is also true for TPH1 and TPH2 gene expression as well as for functional features, such as self-renewal and multipotency, and for functional results (eg, rescue experiments) that were always confirmed throughout subculturing passages. This indicates that, if properly applied, the NSA culture system allows the maintenance of stable functional properties of ANSC over time, excluding the possibility of artifacts because of increasing time in culture (Santa-Olalla *et al*, 2003).

Migration is an important functional feature that is preferentially displayed by lineage-committed precursors and migratory properties could theoretically be exploited for cell-based therapeutics of CNS diseases (Corti *et al*, 2005; Ikegami *et al*, 2005; Prestoz *et al*, 2001; Seki, 2002; Shan *et al*, 2006). We show here for the first time that neural cells dissociated from serially passaged adult hippocampal neurospheres display increased migration activity in response to 5-HT *in vitro*. Plating ANSC in adhesion, in the presence of EGF and FGF2 favors ANSC proliferation and migration, because both mitogens have known pro-migratory activity on neural progenitors *in vivo* (Doetsch *et al*, 2002; Kuhn *et al*, 1997) and *in vitro* (Gritti *et al*, 1995, 1999, 2000). On the contrary, removal of mitogens from the culture medium quickly reduced ANSC proliferation and induced spontaneous differentiation, which results in low migratory activity. In the presence of 5-HT, migratory activity of ANSC is rescued to levels close to those observed in the presence of mitogens and given that no significant differences in cell survival and proliferation in the 6-h experimental time frame (data not shown) were observed, our data suggest that ANSC display a sustained migratory activity in response to 5-HT, and further that 5-HT might regulate different ANSC properties such as proliferation. They also propose a role of 5-HT in the migration of ANSC-derived progeny during neurogenesis *in vivo*.

In conclusion, the data presented here indicate a positive regulatory effect of 5-HT on proliferation, survival, and migration properties of ANSC. This offers ANSC as a reliable *in vitro* system to study the impact that neurotransmitters and drugs might have on neurogenesis, and support their potential relevance as a screening tool for the development of therapeutic approaches to treat affective disorders (Dranovsky and Hen, 2006; Kempermann and Kronenberg, 2003; Warner-Schmidt and Duman, 2006).

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DISCLOSURE

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