Journal of Neurochemistry

Submitted Version. DOI: 10.1111/jnc.13962

Serotonin receptor 5-HT7 increases the density of dendritic spines and facilitates synaptogenesis in forebrain neurons

Journal:	*Journal of Neurochemistry*
Manuscript ID	JNC-2016-0664.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Speranza, Luisa; Institute of Genetics and Biophysics "Adriano Buzzati Traverso", CNR, Naples, IT Labus, Josephine; Medizinische Hochschule Hannover, Cellular Neurophysiology Volpicelli, Floriana; University of Naples Federico II, Department of Pharmacy; Institute of Genetics and Biophysics "Adriano Buzzati-Traverso", Developmental Neurobiology Guseva, Daria; Hannover Medical School, Hannover, Germany lacivita, enza; Department of Pharmacy, University of Bari "A. Moro", Bari, IT Leopoldo, Marcello; Department of Pharmacy, University of Bari "A. Moro", Bari, IT Bellenchi, Gian Carlo; Insitute of Genetics and Biophysics "Adriano Buzzati-Traverso", Department of Biomedicine, CNR di Porzio, Umberto; Inst. Genetices & Biophysics, Develp. Neurobiol. Bijata, Monika; Medizinische Hochschule Hannover, Cellular Neurophysiology Perrone-Capano , Carla; Institute of Genetics and Biophysics "Adriano Buzzati Traverso", CNR, Naples, IT Ponimaskin, Evgeni; Medical school Hannover, Center of Physiology
Area/Section:	Signal Transduction & Synaptic Transmission
Keywords:	5-HT7 receptor, neurite outgrowth, dendritic spines, synaptogenesis, Cdk5, Cdc42

SCHOLARONE™
Manuscripts

Serotonin receptor 5-HT7 increases the density of dendritic spines and facilitates synaptogenesis in forebrain neurons

Speranza Luisa[a,b,c#], Labus Josephine[b#], Volpicelli Floriana[d], Guseva Daria[b], Lacivita Enza[e], Leopoldo Marcello[e], Bellenchi Gian Carlo[a,f], di Porzio Umberto[a], Bijata Monika[b], Perrone-Capano Carla[a,d*], Ponimaskin Evgeni[b*]

(a) Institute of Genetics and Biophysics "Adriano Buzzati Traverso", CNR, Naples, IT; (b) Cellular Neurophysiology, Hannover Medical School, Germany; (c) Department of Neuroscience Albert Einstein College of Medicine Department of Pharmacy, New York, USA; (d) University of Naples Federico II, Naples, IT; (e) Department of Pharmacy, University of Bari "A. Moro", Bari, IT; (f) IRCCS Neuromed, Pozzilli (IS), IT.

#These authors contributed equally to this work

*Equally contributing corresponding authors

Corresponding authors: Ponimaskin Evgeni, Cellular Neurophysiology/OE4230, Hannover Medical School. Carl-Neuberg-Str. 1, 30625 Hannover, Germany. Phone: +49-511-5324858, E-mail: Ponimaskin.evgeni@mh-hannover.de

Perrone-Capano Carla, University of Naples Federico II, Department of Pharmacy, Via D. Montesano 49, 80131 Naples, Italy. Phone: 39-081-2535081, Fax: 39- 081-678107, E-mail: perrone@unina.it

For Peer Review

ABSTRACT

Precise control of dendritic spine density and synapse formation is critical for normal and pathological brain functions. Therefore, signaling pathways influencing dendrite outgrowth and remodeling remain a subject of extensive investigations. Here we report that prolonged activation of the serotonin 5-HT7 receptor (5-HT7R) with selective agonist LP-211 promotes formation of dendritic spines and facilitates synaptogenesis in postnatal cortical and striatal neurons. Critical role of 5-HT7R in neuronal morphogenesis was confirmed by analysis of neurons isolated from 5-HT7R-deficient mice and by pharmacological inactivation of the receptor. Acute activation of 5-HT7R results in pronounced neurite elongation in postnatal striatal and cortical neurons, thus extending previous data on the morphogenic role of 5-HT7R in embryonic and hippocampal neurons. We also observed decreased number of spines in neurons with either genetically (*i.e.* 5-HT7R-KO) or pharmacologically (*i.e.* antagonist treatment) blocked 5-HT7R, suggesting that constitutive 5-HT7R activity is critically involved in the spinogenesis. Moreover, cyclin-dependent kinase 5 (Cdk5) and small GTPase Cdc42 were identified as important downstream effectors mediating morphogenic effects of 5-HT7R in neurons. Altogether, our data suggest that the 5-HT7R-mediated structural reorganization during the postnatal development might have a crucial role for the development and plasticity of forebrain areas such as cortex and striatum, and thereby can be implicated in regulation of the higher cognitive functions.

Abbreviations: 5-HT: 5-hydroxytryptamine; 5-HT7R: serotonin receptor 7; Cdk5: cyclin-dependent kinase 5; CTRL: vehicle-treated control; CTX: cortex; DIV: days *in vitro*; E: embryonic age; KO: knock-out; P: postnatal day; STR: striatum; WT: wild-type.

For Peer Review

KEY WORDS

5-HT7R; neurite outgrowth; dendritic spines; synaptogenesis; Cdk5; Cdc42.

RUNNING TITLE

Morphogenic properties of 5-HT7R in neurons

For Peer Review

Introduction

The most fascinating and important property of the mammalian brain is its ability to adapt in response to behavioral stimuli through synaptic plasticity (Fernandes and Carvalho 2016). This process involves modifications of neuronal circuitry, which can occur: (*i*) by changing the force or the efficacy of synaptic transmission at pre-existing synapses, (*ii*) by modulating the neuronal excitability, or (*iii*) by remodeling of synaptic connections or by pruning of the existing ones. The synaptic remodeling can occur through a rearrangement of neuronal morphology both at presynaptic and postsynaptic sites. The latter structural modifications are mainly supported by the plastic changes of dendritic spines, small protrusions with various shapes and sizes emerging from the dendritic shaft, first described by Ramon y Cajal (Cajal 1988, Sala and Segal 2014). In the mammalian forebrain, dendritic spines form the postsynaptic component of most excitatory synapses. They exhibit a wide range of morphological diversity and change their shape and size continuously, being highly dynamic during early developmental stages as well as in the mature nervous system (Lai and Ip 2013). Spine formation, turnover and morphology are continuously modulated by synaptic activity occurring during memory formation and other adaptive changes of the brain (Fiala *et al.* 2002, Chang *et al.* 2013). Accordingly, spine volumes increase after long term potentiation (LTP), whereas long term depression (LTD) causes spine shrinkage, indicating that remodeling is linked to the synaptic force (Holtmaat and Svoboda 2009). Precise control of dendritic spine morphology and density is critical for normal brain function. Consequently, aberrant spine morphology is often linked to neurological, neurodegenerative and psychiatric disorders (Chang *et al.* 2013). Therefore, the cues and signaling pathways influencing dendrite outgrowth and remodeling still remain a subject of active investigation (Lefebvre *et al.* 2015).

The actin cytoskeleton plays a pivotal role in the formation, elimination, motility and stability of dendritic spines as well as in regulation of their size and shape. Modulation of actin dynamics

For Peer Review

drives the morphological changes in dendritic spines that are associated with alteration in synaptic strength (Lei *et al.* 2016). At the molecular level, there are multiple molecules that regulate the actin polymerization and stabilization, including the members of the Rho family of small GTPases (*e.g.* Rho, Rac, and Cdc42), which in turn modulate phosphorylation of the actin-binding protein cofilin, influencing the dendritic spine's structure and dynamic (Ponimaskin *et al.* 2007, Woolfrey and Srivastava 2016). We have previously demonstrated that activity of small GTPases Cdc42 and RhoA in neurons can be controlled and regulated by the serotonin receptor 7 (5-HT7R) resulting in receptor-mediated changes of neuronal morphology and synaptic functions. These effects are mediated by the coupling of the 5-HT7R with the $G_{\alpha 12}$-protein (Kvachnina *et al.* 2005, Kobe *et al.* 2012). Accordingly, accumulating data suggest an important role for the 5-HT7R in regulation of structural plasticity of brain circuits (Volpicelli *et al.* 2014, Wirth *et al.* 2016). The 5-HT7R is a G protein-coupled receptor broadly expressed in the central nervous system, including spinal cord, thalamus, hypothalamus, hippocampus, prefrontal cortex, striatal complex and amygdala. Its distribution in differently specialized brain regions reflects the key role played by this receptor in controlling diverse neural functions such as circadian rhythms, sleep-wake cycle, thermoregulation, nociception, learning and memory processing (Daubert and Condron 2010, Lesch and Waider 2012). Consistently, aberrant receptor-mediated signaling has been involved in numerous neuropathological processes such as anxiety, schizophrenia, epilepsy, migraine, impulsivity and depression, cognitive and mood dysfunctions, and very recently also neuropathic pain and itch disorders (Leopoldo *et al.* 2011, Matthys *et al.* 2011, Naumenko 2014, Morita *et al.* 2015, Santello and Nevian 2015).

It has been shown that pharmacological stimulation of this receptor enhances neurite outgrowth in embryonic neuronal primary cultures from hippocampus, cortex, and striatum via the activation of signaling transduction pathways that converge on the reorganization of

For Peer Review

cytoskeletal proteins (Speranza *et al.* 2013, Speranza *et al.* 2015). These data propose the 5-HT7R as a part of molecular cascade required for the growth of new synapses and the formation of initial neuronal networks during critical period of embryonic neuronal wiring. On the other hand, the involvement of the 5-HT7R in the morphological remodeling of postnatal neurons has been studied only in hippocampal neurons, where its activation potentiates the formation of dendritic spines, increases neuronal excitability, and modulates synaptic plasticity only during the early postnatal development (Kobe et al. 2012).

In the present study, using neuronal preparation from wild type (WT) and 5-HT7R deficient mice, we analyzed the involvement of 5-HT7R in the structural plasticity of postnatal neurons from forebrain areas (striatum and cortex). In order to evaluate the acute and chronic effects of 5-HT7R activation on neurite outgrowth, dendritic spines development and on synaptogenesis, neurons were treated with the highly selective 5-HT7R agonist LP-211 (Hedlund *et al.* 2010) for several hours or for 3 days, respectively. In addition, we analyzed intracellular signaling pathways underlying 5-HT7R-mediated changes in dendritic spines.

Materials and Methods

Culturing and transfection of striatal and cortical neurons

Mice pups were housed, cared and sacrificed in accordance with the recommendations of the European Commission (EU Directive 2010/63/EU for animal experiments). All the procedures related to animal treatments were approved by Ethic-Scientific Committee for Animal Experiments. Striatal (STR) and cortical (CTX) neurons were prepared from WT and 5-HT7R-KO (Hedlund *et al.*, 2003) C57BL/6J mouse pups. Pups of both sexes were used. Brains were isolated from P1-P3 mice under sterile condition and placed in HBSS (ThermoFisher Scientific, Milan, Italy, Cat.N. 24020-091). STR or CTX areas were dissected from the brain under a stereomicroscope and enzymatically dissociated by incubation for 30 min at 37°C in a papain

For Peer Review

solution (Worthington, 10 U/ml) in DMEM containing 50 mM EDTA, 100 mM $CaCl_2$, 2 mg/ml cysteine and 0.01% pancreatic DNAse. After incubation, enzyme dissociation was blocked by incubation for 5 min at RT with 25 mg/ml albumin, 25 mg/ml trypsin-inhibitor and 10% FBS medium (10% FBS, 50 U/ml penicillin and 50 mg/ml streptomycin). The cells were resuspended in 10% FBS medium and cell concentration was determined on the basis of the total cell count after the trypan blue dye exclusion. Dissociated cells were plated in Neurobasal A medium supplemented with B27 (ThermoFisher Scientific) and 1% FBS, 2 mM Glutamax (ThermoFisher Scientific), 50 U/ml penicillin and 50 mg/ml streptomycin (ThermoFisher Scientific) at a density of 70 x $10^3/cm^2$ onto sterilized 12 mm coverslips or cell culture dishes (Corning) freshly coated with 15 µg/ml of poly-D-Lysine (Sigma-Aldrich, Milan, Italy). On the second day *in vitro* (DIV), and every third DIV, half of the medium was replaced by fresh medium without FBS. Cultures were maintained at 37°C and 5% CO_2 in a humidified incubator for 5-12 days. For each experimental point, cultures were prepared in independent triplicates and experimental points were repeated using independent cell preparations.

For dendritic spines morphological analysis, neurons were transfected at DIV 7 with 1 µg of pcDNA3.1 vector encoding GFP by using Lipofectamine 2000 reagent (ThermoFisher Scientific), according to the manufacture's protocol. Cells were fixed after 3 days of pharmacological treatment (from DIV 10 to DIV 12) during which every day the culture medium was completely changed and replaced with fresh medium supplemented with drug.

Drugs and reagents

For the treatment of cell cultures we used 100 nM of the selective 5-HT7R agonist, LP-211 (provided by M. Leopoldo, University of Bari, Italy), 100 nM of the 5-HT7R antagonist SB-269970 (Tocris, Milan, Italy) alone or in combination with LP-211. CdK5 inhibitor roscovitine (Sigma-Aldrich) was used at the final concentration of 20 µM. A selective inhibitor of Cdc42 ZCL 278 (Tocris, Milan, Italy) was used at final concentration of 50 µM. Drugs were added to

For Peer Review

cultures at the DIV as indicated in the results section or in the figure legends and incubated for appropriate time.

RNA isolation and RT-PCR analyses

RNA extraction, reverse transcription and SYBR Green real time-PCR reactions were performed as previously described (Speranza *et al.*, 2013) from P3 STR and CTX primary cultures collected at DIV 4 and DIV 12. The analyses were carried out in triplicates for each experimental point. Quantitative real time PCR was performed by using the following primer sets:

5-HT7R: Fw, 5' GCGGTCATGCCTTTCGTTAGT 3'; rev, 5' GGCGATGAAGACGTTGCAG 3'. Hypoxanthine phosphoribosyltransferase (HPRT): Fw, 5' TGGGAGGCCATCACATTGT 3' ; rev, 5' AATCCAGCAGGTCAGCAAAGA 3'.

Quantitative analysis of gene expression levels was performed by the comparative threshold cycle (*CT*) method (Schmittgen and Livak, 2008), using HPRT as an internal control gene. The relative expression level of the gene of interest was expressed as $2^{-\Delta CT}$ where $\Delta CT = CT$ gene of interest - *CT* HPRT.

Immunofluorescence and morphological analyses

For morphological analyses, postnatal cultures were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 20 min at RT, washed three times in PBS, and permeabilized for 15 min in PBS containing 0.3% Triton-X-100. The neurons were treated for 1 h at RT in blocking solution (3% BSA in PBS) and then incubated overnight at 4°C with the primary antibody in PBS containing 1% BSA. The monoclonal antibody against neuron specific class III ß-tubulin (Tuj1, Sigma-Aldrich T8660, 1:500) was used to stain neurons. Cells were then washed in PBS and incubated for 2 h at RT with fluorescent-labeled secondary antibodies (Alexa Fluor 594, 1:400, ThermoFisher Scientific) in PBS with 1% BSA. After washing, cells were stained with DAPI (nuclear stain, 1:1,000) for 10 min at RT and mounted on coverslip

with oil mounting solution (Mowiol). As negative controls, cells processed without primary antibody were used.

To evaluate neurite length, fluorescent signals from Tuj1 stained neurons were detected with a microscope (Leica DM6000B) equipped with a 20x objective. Images were acquired with high-resolution camera using the software Leica Application Suite, and analyzed by the image-processing software Image J. The length of neurites was measured as described in Speranza *et al.* 2015). A total of 15-20 fields for each cell-culture condition was used from at least three independent culture wells.

For dendritic spines analysis, images of GFP-transfected neurons were acquired on Zeiss LSM780 confocal microscope using a 40x water and 63x oil immersion objective. To perform quantitative spine analysis, proximal dendritic segments were analyzed from both primary and secondary dendrites. As expected at the initial stages of spinogenesis, most of the dendritic protrusions were filopodia-like processes (Hering and Sheng 2001), while more mature spines with a well-defined morphology (thin or mushroom) were less than 1%. Dendritic protrusions with a length of 5 -25 μm were counted and spine density was expressed as number of spines per 50 μm of dendritic length. A blind analysis was carried out in all cases to avoid any subjective influences during the measurements. For easy comparison of the results among various cell preparations, data were expressed as percentage of the average CTRL. Representative images were depicted as maximum intensity projection.

Immunofluorescence-based assay to quantify synapse number

Neuronal cultures were grown on 12 mm glass coverslips in 24-well plates coated with poly-D-lysine (Sigma-Aldrich) as described above. At DIV 10 cells were treated for 3 days with 100 nM LP-211. During this treatment, the culture medium was replaced every day with fresh medium supplemented with the drug. Cells were then fixed for 10 min with 4% PFA, permeabilized for 15 min with 0.3% Triton-X-100 in PBS, blocked for 30 min with 10% NGS

For Peer Review

(normal goat serum), 1% BSA, 0.05% Triton-X-100 in PBS and incubated overnight at 4°C with rabbit anti-synaptophysin (1:200, Millipore, AB9272, Milan, Italy) and mouse anti-PSD95 (1:200, Millipore, MABN68) antibodies, diluted in antibody buffer (3% BSA, PBS). After that, goat anti-mouse Alexa-594 (ThermoFisher Scientific) and goat anti-rabbit Alexa-488 (ThermoFisher Scientific) conjugated secondary antibodies were diluted 1:200 in antibody solution and were applied for 2h at RT. After incubation for 10 min with DAPI, coverslips were mounted onto glass slides with Mowiol. Images were acquired using the fluorescent microscope Nikon A1 with a 60x oil immersion objective. To analyze the number of synaptic clusters, the co-localization of synaptophysin- and PSD95-positive puncta was calculated along the dendrite with the plugins "Colocalization" and "Colocalization finder" of the ImageJ software. At least 20 randomly collected images were used for each treatment, and the number of synaptophysin/PSD95-positive puncta was calculated per 50 µm of dendritic length.

Direct measurement of Cdc42 activation

Pull-down experiments were performed as described previously (Bijata *et al*, 2015). Shortly, cortical neurons were homogenized in lysis buffer [25 mM HEPES (pH 7.5), 150 mM NaCl, 1% Non-idet P-40, 10 mM MgCl2, 1 mM ethylenediaminetetraacetic acid (EDTA), and 2% glycerol] and centrifuged at 14,000 × g for 10 min. The cell extracts (500 ug protein) were incubated with GST-PAK-PBD (Cell BioLabs) fusion protein that had been conjugated with glutathione beads at 4°C overnight and washed three times with lysis buffer. GST-PAK-PBD-bound Cdc42 was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently immunoblotted with Cdc42-specific antibody (1:500; 11A11, Cell Signaling).

Design and data analyses

To evaluate consequences of different pharmacological treatments, statistical analysis was performed on primary neuronal cultures and compared with control neurons from the same

For Peer Review

batch of dissociated cells, treated in parallel with vehicle alone (CTRL). Significance of differences was assessed by One-Way ANOVA followed by Tukey post-hoc test. Significance threshold was set at $p < 0.05$. Statistical analysis was performed using GraphPad Prism 5 Software.

Results

Acute stimulation of 5-HT7R facilitates neurite elongation in cortical and striatal neurons

To investigate the modulatory role of 5-HT7R on neuronal morphology, we prepared primary cultures of striatal (STR) and cortical (CTX) neurons from WT and 5-HT7R-KO mice at postnatal (P) days P1-P3. Both cultures were stimulated at DIV 4 with the 5-HT7R selective agonist LP-211 (LP, 100 nM) for various time intervals (1, 2 and 6 h). After that, neurons were labeled with the neuron-specific anti-ßIII-tubulin antibody (TuJ1) and evaluated for morphological changes. As shown in Fig. 1A, 1 h stimulation of striatal WT neurons with 100 nM LP had no effect on neurite length, whereas agonist-stimulation for 2 h resulted in a significant enhancement of neurite length (+ 37%) relative to the vehicle-treated control cultures (CTRL). This effect was mediated by the 5-HT7R, as co-treatment of the cells with 100 nM LP and the 5-HT7R selective antagonist SB-269970 completely abolished neurite elongation, while addition of the antagonist alone for 2 h had no effect. The effect of 5-HT7R stimulation on neurite outgrowth remained significant also after 6 h of treatment with the agonist. In contrast, no effect of LP was observed in striatal neurons isolated from the 5-HT7R deficient mice at all time points analyzed (Fig. 1B), further confirming that neurite elongation obtained in WT neurons was mediated by the 5-HT7R.

To investigate whether the 5-HT7R-mediated neurite outgrowth could occur in neurons from other forebrain areas, we performed similar experiments in primary cortical neurons isolated from WT and 5-HT7R-KO animals. In accordance with results obtained in striatal neurons,

For Peer Review

pharmacological stimulation of the 5-HT7R significantly increased neurite elongation in cortical neurons from WT animals after 2 h compared to CTRL (+24%), and this effect persisted for up to 6h (Fig. 2A). This effect was 5-HT7R specific, since no effect of LP treatment on neurite elongation was observed in neurons co-treated with SB-269970, nor in cortical cultures isolated from 5-HT7R-KO mice (Fig. 2B).

Noteworthy that the primary branching, evaluated as total number of neurites originating directly from soma, was not affected by the stimulation of the 5-HT7R with LP-211 both in striatal as well as cortical neurons isolated from WT mice. In contrast, primary branching was reduced in CTRL and LP-treated cultures prepared from 5-HT7R-KO mice. This decrease was statistically significant in striatal cultures. Similar tendency, although not statistically significant, was observed in cortical neurons (Fig. S1).

It is also noteworthy that WT striatal and cortical untreated cultures show a basal receptor-independent neurite elongation occurring without agonist stimulation: vehicle-treated cultures analyzed after 6 h show significant increased neurite length in both striatal and cortical neurons (+23% and +17%, respectively) compared with vehicle-treated cultures investigated after 1h (Fig. 1A and 2A). Since a time-dependent increase in neurite length was observed also in cultures isolated from the 5-HT7R deficient mice (+27% and +10%, respectively, Fig. 1B and 2B), these results suggest that the neuronal machinery responsible for the basal neurite outgrowth is partially inhibited, but not abolished in absence of the 5-HT7R.

Altogether, these results demonstrate that activation of the 5-HT7R stimulates neurite elongation in postnatal striatal and cortical neurons, thus extending previous data on morphogenic role of 5-HT7R obtained on embryonic and hippocampal neurons (Kobe *et al.* 2012, Speranza *et al.* 2013, Speranza *et al.* 2015).

For Peer Review

Chronic 5-HT7R stimulation increases the number of dendritic protrusions in postnatal striatal and cortical neurons

To investigate the role of the 5-HT7R-mediated signaling in spinogenesis of striatal and cortical neurons, we selected a time window from DIV10 to DIV12, when well-defined synapses are already formed (Kobe *et al*. 2012). The level of the transcripts encoding the 5-HT7R is quite stable during the *in vitro* development, as neurons at DIV 4 and DIV 12 show similar mRNA expression levels (Fig. S2). To monitor receptor-mediated morphological changes, GFP-transfected neurons were incubated with a low concentration of the 5-HT7R selective agonist LP (100 nM) during the last 3 days, followed by microscopic analysis of living cells. As expected for these early stages of spinogenesis, most of the dendritic protrusions represent filopodia-like protrusions, while more developed thin, stubby and mushroom-shaped spines are relative rare. Dendritic filopodia are believed to draw the presynaptic contact to the dendrite, leading to the formation of synapses from which mature spines subsequently emerge (Hering and Sheng 2001). As shown in Figure 3A, agonist treatment in striatal WT cultures substantially increased the number of dendritic protrusions per 50 µm dendrite compared to CTRL (+22%). These morphogenic effects were receptor-specific because they were completely inhibited by the selective 5-HT7R antagonist SB-269970 . Noteworthy, treatment of neurons with antagonist alone resulted in a significantly reduced number of dendritic spines compared to CTRL (-26%). Similar results were obtained for co-application of agonist and the antagonist (Fig. 3A). These findings suggest that the basal, agonist-independent activity of 5-HT7R leads to an increase in spine density of striatal neurons and this effect can be further enhanced by receptor activation. Confirming this assumption, the number of dendritic protrusions in non-treated 5-HT7R-KO striatal neurons was significantly reduced compared to WT neurons, both under basal condition (-38%) as well as after agonist stimulation (-57%). As expected, the density of dendritic spines in 5-HT7R-KO cultures was unchanged by agonist stimulation. In addition, a statistical analysis

For Peer Review

performed on striatal neurons isolated from 5-HT7R-deficient mice revealed that the number of dendritic protrusions was indistinguishable between LP-treated and non-treated neurons (Fig. 3A). Treatment of 5-HT7R-KO striatal neurons with SB-269970 alone, or in combination with LP-211 had no effect (Fig. 3A).

Similar results were also obtained in cortical postnatal cultures: the number of dendritic spines per 50 μm of dendritic length was significantly increased in LP-treated WT neurons compared to CTRL (+19%, Fig. 4A). As observed in striatal neurons, density of dendritic protrusions was significantly reduced in cortical neurons isolated from 5-HT7R-deficient mice both under basal condition (- 37%) as well as after LP treatment (- 28%), suggesting that the lack of the receptor impairs, but does not abolish the development of dendritic spines. Constitutive activity of the 5-HT7R receptor was also preserved in cortical neurons, since treatment of WT cultures with SB-269970 significantly reduced the number of dendritic spines compared to WT CTRL (-30%). Accordingly, number of dendritic spines was reduced in neurons prepared from 5-HT7R-deficient mice in comparison with the WT neurons (Fig. 4A).

Taken together, these data show that chronic stimulation of 5-HT7R strongly enhances the number of dendritic protrusions during postnatal development in both striatal and cortical neurons, and that constitutive activity of the 5-HT7R plays an important role in spinogenesis.

Stimulation of the 5-HT7R increases the number of synaptic contacts

The observation that chronic stimulation of 5-HT7R enhances the density of dendritic spines prompted us to investigate whether these morphological changes are accompanied by the formation of new synaptic connections. To this aim, P1 striatal neuronal cultures were treated with LP from DIV 10 to DIV 12. After that, the expression of synaptophysin and postsynaptic density protein (PSD-95) used as pre- and post-synaptic markers, respectively, was analyzed

For Peer Review

by immunostaining (De Camilli *et al.* 1990, Garner *et al.* 2000, De Paola *et al.* 2003). Finally, the number of co-localized puncta was scored in control and LP-treated cultures.

As shown in figure 5, the synapses formed in both LP-treated and control neurons appeared to be structurally intact, as defined by the tight co-localization of PSD-95 and synaptophysin (Fig. 5B). More importantly, treatment of neurons with LP significantly increased the number of synaptophysin/PSD95-positive puncta (+50%, Fig. 5A), indicating that chronic activation of the 5-HT7R strongly stimulates synaptogenesis.

Signal transduction pathways involved in 5-HT7R-mediated spine formation

Multiple signaling molecules are known to be involved in regulation of actin dynamics, polymerization and stabilization in neurons. Among these, Cyclin-dependent kinase 5 (Cdk5) and the small GTPase Cdc42 (Kawauchi 2014, Woolfrey and Srivastava 2016) play an important role in modulation of dendritic spines morphology. Since these signaling molecules have been suggested as down-stream effectors of 5-HT7R (Kobe *et al*. 2012, Speranza *et al*. 2015, Wirth *et al*. 2016), we hypothesized that they could also be involved in 5-HTR7-mediated dendritic spine formation.

To verify this hypothesis, striatal neurons transfected with GFP were treated at DIV 10 with LP (100 nM) for 3 days in presence or in absence of the Cdk5 inhibitor roscovitine (20 μM). Noteworthy, roscovitine administration not only completely blocked dendritic spine formation induced by LP, but also reduced spine density compared to the control conditions (Fig. 6). Statistical analysis revealed that treatment of neurons with roscovitine alone results in a significant reduction in number of dendritic spines compared to CTRL (-35%). Interestingly, parallel application of LP does not overcome inhibitory effect of roscovitine. This data suggests that Cdk5 acts as a down-stream effector in 5-HT7R-mediated spine formation and can be activated already by the constitutive receptor activity.

Similarly, co-application of Cdc42 inhibitor ZCL 278 (50 μM) and LP not only blocked the LP-mediated increase in spine formation, but also resulted in decreased number of dendritic spines compared to CTRL (-30%). It is noteworthy that treatment with ZCL alone also decreased the spine number below the basal level obtained in CTRL (-31%, Fig. 6). To provide direct evidence for 5-HT7R-mediated activation of Cdc42, we used the Cdc42-binding domain of Cdc42 effector, PAK serine/threonine kinase, to affinity precipitate active Cdc42 as a direct readout for receptor-mediated Cdc42 activation (Kvachnina *et al*., 2005). Stimulation of the 5-HT7R in WT cortical neurons induced an increase in Cdc42 activity, while this effect was completely blocked by the pre-treatment with SB (Fig. S3). In addition, no LP-211 mediated Cdc42 activation was obtained in 5-HT7R KO cells (Fig. S3).This data demonstrates that Cdc42 acting as 5-HT7R down-stream effector might mediate the increase in spine number obtained after receptor stimulation.

To verify whether constitutive activity of 5-HT7R is sufficient to induce the Cdk5/Cdc42 signaling pathway we analyzed spine density in 5-HT7R-KO neurons treated with roscovitine and ZCL278. In contrast to WT neurons, inhibition of Cdk5 and Cdc42 in 5-HT7R-KO neurons had no effect on spinogenesis. Furthermore, co-application with 5-HT7R agonist LP did not influence spine density in these cells (Fig. 6).

Combined results of these experiments suggest that CdK5 and Cdc42 activity are required to maintain the appropriate number of spines observed in striatal neurons.

Discussion

The sprouting and outgrowth of neurites followed by the formation of axons and dendrites is an initial critical process in the early stage of neurodevelopment and differentiation of the CNS that directs brain connectivity (Cheng and Poo 2012, Lefebvre *et al*. 2015, Takano *et al.* 2015). The identification and characterization of signalling pathways influencing the remodelling of

For Peer Review

neuronal morphology and synaptic connections is still a subject of extensive investigation (Lei *et al*. 2016). Recently it has been shown that the activation of the 5-HT7R has a morphogenic function during embryonic and early postnatal development thus playing a prominent role in regulating the neuronal cytoarchitecture (Volpicelli *et al*. 2014, Wirth *et al*. 2016).

In the present study, using cultured postnatal mouse striatal and cortical neurons from WT and 5-HT7R-deficient mice, we evaluated the morphological effects of acute and chronic 5-HT7R pharmacological stimulation on neurite outgrowth, dendritic spine development and synaptogenesis. We also investigated the underlying signalling pathways. To selectively stimulate 5-HT7R, we used highly potent selective agonist LP-211 (Hedlund *et al*. 2010). Our results show that activation of the 5-HT7R increases neurite elongation as well as the number of dendritic protrusions, and promotes formation of morphologically intact synapses in postnatal cortical and striatal neurons from mouse. The data on neurite outgrowth and spinogenesis are in line with our previous results obtained in early embryonic neuronal cultures (Speranza et al. 2013, Speranza *et al*. 2015) as well as in hippocampal neurons during the early postnatal period (Kvachnina *et al*. 2005, Kobe *et al*. 2012). The increase in spine density observed in striatal and cortical postnatal neurons indicates that the morphogenic effects mediated by the 5-HT7R are also present at the later developmental stages, when polarization of neurons has already occurred. In accord with this view, we have recently shown that axonal growth is also enhanced by 5-HT7R activation in hippocampal neurons (Speranza et al. 2015). It thus appears that this receptor can act at early and late developmental stages, shaping neuronal morphology and neural circuit assembly in various brain areas.

We demonstrated that prolonged stimulation of the 5-HT7R leads to a pronounced increase in the number of dendritic protrusions in both striatal and cortical neurons. It has been proposed that early dendritic protrusions have a highly dynamic behaviour, suggesting that they play an exploratory role, presumably to actively initiate physical contact with nearby axons followed

For Peer Review

by synapse formation (Mattila and Lappalainen 2008). This hypothesis is supported by data showing that synaptogenesis is impaired following manipulations that alter spine dynamics (Kayser *et al.* 2008, Mattila and Lappalainen 2008). Accordingly, we found that the number of newly formed synapses was strongly increased after selective stimulation of the 5-HT7R. Altogether, these data support the hypothesis that modulation of the number of dendritic protrusions is associated with modifications of synaptic contacts. Results obtained in the present study are also consistent with previous data demonstrating that activation of 5-HT7R with a non-selective agonist (5-CT) promotes synaptogenesis and enhances synaptic activity in hippocampal neurons (Kobe *et al.* 2012). However, the expression level of 5-HT7R in hippocampus has been shown to progressively decrease during the postnatal development. Consistently, stimulatory effects of the 5-HT7R-mediated signaling on spine formation and synaptogenesis in hippocampal neurons were restricted to early postnatal development stages, thus influencing the formation of basal neuronal connections during these stages (Kobe *et al.* 2012). In contrast, expression of the 5-HT7R in cortex and striatum remains stable during the whole postnatal development (Vizuete *et al.* 1997, Adriani *et al.* 2006, Leo *et al.* 2009), suggesting that 5-HT7R can participate in reorganization of neuronal networks and modulation of neural plasticity also during the later developmental stages and in adulthood. This indication was indirectly supported by the recent observation that administration of the 5-HT7R agonist LP-211 in adolescent rats leads to increased neural dendritic arborization in the nucleus accumbens, as well as increased functional connectivity between different forebrain networks that are proposed to be involved in anxiety-related behavior (Canese *et al.* 2015).

To demonstrate that the morphogenic effects depend on 5-HT7R, we used neuronal cultures obtained from the 5-HT7R-deficient mice (Hedlund *et al.* 2003) as well as WT cultures treated with a highly selective 5-HT7R antagonist SB-269970 (Hagan *et al.* 2000). The results of these experiments not only confirmed the selective involvement of the receptor in the morphological

For Peer Review

modifications observed, but also provided us with unexpected and novel information. Indeed, we observed a lower number of spines in non-treated neurons with either genetically (i.e. 5-HT7R-KO) or pharmacologically (i.e. SB-269970 treatment) blocked 5-HT7R, suggesting that agonist-independent, constitutive 5-HT7R activity is critically involved in the formation and/or outgrowth of the "basal" number of dendritic protrusions. This hypothesis has been confirmed by the observation that the lack of the receptor in 5-HT7R-KO cultures treated with the selective antagonist SB-269970 does not affect the density of dendritic protrusions.

Constitutive activity has been observed for multiple G-protein coupled receptors and implies the capacity of receptors to convert from the inactive to the active form in the absence of agonist (Cotecchia *et al.* 1990, Rosenbaum *et al.* 2009). The constitutive activity of a native receptor is an important pharmacological characteristic because it might explain partly its roles in physiological and pathological conditions, as well as the effect of drugs classified as inverse agonists (Seifert and Wenzel-Seifert 2002). Our combined data indicate that the constitutive activity of the 5-HT7R modulates dendritic spine formation, without affecting the neurite outgrowth. One possible explanation is that the effects mediated by the blockade of constitutive 5-HT7R activity by SB might require longer times in case of neurite elongation, while spinogenesis is a more quick process. Alternatively, 5-HT7R constitutive activity might be specifically involved in activation of signalling pathways responsible for spinogenesis, while receptor stimulation can modulate both neurite outgrowth as well as spinogenesis. Indeed, stimulation of 5-HT7R is known to promote the mTOR-mediated phosphorylation of p70S6 kinase, which in turn modulates the expressions of two proteins involved in spinogenesis: CamKII and Shank3 (Bhattacharya *et al.*, 2012). Future experiments will clarify how, where and when the constitutive receptor activity could affect density of dendritic spines *in vivo* during development.

For Peer Review

Proper wiring of the brain during development is critical for cognition and memory, and growing evidence indicates that a key cellular correlate of information encoding is the modulation of dendritic spines (Tau and Peterson 2010, Yuste 2011). From this point of view, it is tempting to speculate that the learning defects observed in 5-HT7R-KO mice (Roberts and Hedlund 2012) might be associated to defective dendritic spine morphology due to lack of the receptor. It has recently been shown that activation of the 5-HT7R in the forebrain can reverse the dysfunction of dendritic integration induced by neuropathic pain (Santello and Nevian 2015). According to results obtained in the present study, this rescue might be linked to 5-HT7R-mediated modulation of dendritic spines. Besides the treatment of chronic pain, the 5-HT7R was suggested as a potential therapeutic target for treatment of neurodevelopmental diseases associated with abnormal CNS connectivity. Indeed, successful rescue of functional and behavioral deficits observed in mouse models of Fragile X syndrome and Rett syndrome has been observed following stimulation of the 5-HT7R (Costa *et al.* 2012, De Filippis *et al.* 2015). Also in this case, therapeutic effect of 5-HT7R can be explained by receptor-mediated remodeling of synaptic connections.

Our work also unravels involvement of Cdk5 and CdC42 activity for the 5-HT7R-mediated morphogenic and synaptogenic effects. It has been recently shown that dendritic spine formation and neuronal maintenance require Cdk5 activity (Mita *et al.* 2016) regulated by its association with the p35 cofactor. The Cdk5/p35 can also regulate spatial learning and memory, further confirming that molecules affecting dendritic spine morphology and density are critical for normal brain function and synaptic plasticity (Kawauchi 2014, Mishiba *et al.* 2014). Interestingly, mice in which Cdk5/p35 activity was deregulated during development show alteration of meso-cortico-limbic circuitry and changes in a locomotor profile and pharmacological responses reminiscent of ADHD (Drerup *et al.* 2010), a pathology whose symptoms are associated to altered connectivity of neural circuits (Hong *et al.* 2014). Molecular

For Peer Review

mechanisms underlying interplay between 5-HT7R and Cdk5 could include direct interaction between these molecules as it has been recently shown for the 5-HT6R. Within this complex, 5-HT6R activates Cdk5 through an mTOR- and cAMP-independent mechanism, initiating neurite outgrowth via the small GTPase Cdc42 in cultured hippocampal and striatal neurons (Duhr *et al.* 2014). Future studies will be therefore needed to evaluate the contribution of 5-HT7R/Cdk5 complex for receptor-mediated spinogenesis.

Another molecule identified as a potential downstream effector of 5-HT7R is Cdc42. This protein belongs to the family of small GTPases that have emerged as key regulators of structural plasticity in neurons, linking extracellular signals with the actin cytoskeleton reorganization. We have previously shown that 5-HT7R can activate not only G_s, but also heterotrimeric G_{12} protein. Prominent downstream effectors of the G_{12} protein are members of the Rho family of small GTPases, including RhoA, Rac1 and Cdc42 (Jaffe and Hall 2005). We have also demonstrated that small GTPases RhoA and Cdc42 represent the main downstream effectors of the 5-HT7R signalling and found that the major functional effects in hippocampal neurons are mediated by the activation of the Cdc42 (Kvachnina *et al*. 2005, Kobe *et al*. 2012). Therefore, Cdc42 is a promising candidate to modulate dendritic spines and to regulate structure and function of synapses in response to 5-HT7R stimulation in striatal and cortical neurons.

Taken together, our data demonstrate a key role of the constitutive and agonist-mediated 5-HT7R activity for regulation of dendritic spine formation and synaptogenesis in postnatal forebrain neurons, suggesting that this receptor might be a part of the molecular cascade required for the establishment and maintenance of connectivity within neuronal networks.

In addition, the morphogenic properties of 5-HT7R obtained in the present study may be partly related to its hetero-oligomerisation with other GPCRs. Our previous study show hetero-dimerisation between 5-HT1A and 5-HT7 receptors (Renner *et al*., 2012). Functionally, among other effects hetero-dimerisation initiates agonist-mediated internalisation of 5-HT1AR, which

For Peer Review

is highly resistant to internalisation when expressed alone. Upon internalisation, 5-HT1AR can activate β-arrestin-mediated stimulation of the mitogen-activated protein kinase (MAPK) in a G protein-independent manner (Renner et al., 2012). Thus, 5-HT7R can indirectly influence activation of distinct Erk-mediated pathways involved in modulation of neuronal morphology (i.e. G protein-dependent or -independent) via the relative amount of 5-HT7R participating in hetero-dimers. It will be thus interesting to know how dimerization influences the morphogenic properties of 5-HT7R in the future studies.

Acknowledgments

This work was supported by DFG grant Po732; and DFG Excellence Cluster Rebirth to E.P., by FIRB-RBIN062YH4 to U.d.P. and by "Finanziamento Ricerca di Ateneo" to C.P.C. We thank FEBS and GLISTEN-COST action for supporting short-term scientific missions of L.S. in the Hannover Medical School. We are grateful to the Integrated Microscopy Facility of the Institute of Genetics and Biophysics "Adriano Buzzati Traverso", CNR, Naples, IT. We are grateful to Prof. M. Crispino, Dr. D. Viggiano and Prof. G. Augusti-Tocco for helpful discussions and feedback on the manuscript. The authors have no conflict of interest.

Figure legends

Fig. 1 Acute pharmacological stimulation of the 5-HT7R increases neurite outgrowth in striatal postnatal cultures. Striatal primary neuronal cultures from P3 WT (A) and 5-HT7R-KO (B) mice were treated with vehicle (CTRL) or with the selective 5-HT7R agonist LP-211 (LP, 100 nM), alone or in combination with the selective 5-HT7R antagonist SB269970 (SB, 100 nM; dotted bars) at various time points (1-6 h), as indicated. Neurite length was measured in cells stained with anti-Tuj1 antibody, and expressed as μm. For each experimental point, cultures were prepared at least in independent triplicates. The bars represent means ± SEM from

For Peer Review

randomly selected fields for each cell culture condition (n=63 cells for WT and n=54 cells for 5-HT7R-KO). The panels on the right display representative images of CTRL and LP-treated striatal neurons immunostained with the neuronal marker Tuj1 (red) and counterstained with the nuclear marker DAPI (blue; magnification 20x).

Asterisk (*): values significantly different from the respective CTRL by one-way ANOVA followed by Tukey post-hoc test ($p < 0.05$). Hash mark (#): values significantly different from each other by one-way ANOVA followed by Tukey post-hoc test ($p < 0.05$).

Fig. 2 Acute pharmacological stimulation of the 5-HT7R enhances neurite length in cortical postnatal cultures. Cortical primary neuronal cultures from P3 WT (A) and 5-HT7R-KO (B) mice were treated with vehicle (CTRL) or with the selective 5-HT7R agonist LP-211 (LP, 100 nM) alone or in combination with the selective 5-HT7R antagonist SB269970 (SB, 100 nM; dotted bars) at various time points, as indicated below the bars. Neurite length was measured on cells stained with anti-Tuj1 antibody, and expressed as μm. For each experimental point, cultures were prepared at least in independent triplicates. The bars represent means ± SEM from randomly selected fields for each cell culture condition (n=39 cells or WT and n=48 cells for 5-HT7R-KO). The panels on the right display representative images of CTRL and LP-treated cortical neurons immunostained with the neuronal marker Tuj1 (red) and counterstained with the nuclear marker DAPI (blue; magnification 20x). Asterisk (*): values significantly different from the respective CTRL by one-way ANOVA followed by Tukey post-hoc test ($p < 0.01$). Hash mark (#):values significantly different from each other by one-way ANOVA followed by Tukey post-hoc test ($p < 0.05$).

Fig. 3 Chronic 5-HT7R activation stimulates formation of dendritic protrusions in postnatal striatal neurons. (A) Density of dendritic spines (average of the total number of dendritic

For Peer Review

protrusions/50 μm dendrite) in striatal neuronal primary cultures, expressed as percent values of control cells. Cells from WT and 5-HT7R-KO mice were transfected with GFP-encoding plasmid at DIV 7 and then treated with vehicle (CTRL) or LP-211 (LP, 100 nM), alone or in combination with the selective 5-HT7R antagonist SB269970 (SB, 100 nM; dotted bars) for three days (from DIV 10 to DIV 12). For each experimental point, cultures were prepared at least in independent triplicates. The bars represent means ± SEM from randomly selected fields for each cell culture condition (n = 57 for WT and n = 66 for 5-HT7R-KO neurons). Asterisk (*): values significantly different from the respective CTRL by one-way ANOVA followed by Tukey post-hoc test ($p < 0.01$). Hash mark (#): values significantly different from each other by one-way ANOVA followed by Tukey post-hoc test ($p < 0.05$). (B) Representative images of WT and 5-HT7-KO striatal neurons treated with vehicle or with LP, SB and LP+SB. White boxes indicate the area of enlargement. Scale bar 20 μm.

Fig. 4 Chronic 5-HT7R activation stimulates formation of dendritic protrusions in postnatal cortical neurons. (A) Density of dendritic spines (average of the total number of dendritic protrusions/ 50 μm dendrite) in cortical primary neuronal cultures, expressed as percent values of control cells. Cells from WT and 5-HT7R-KO mice were transfected with GFP-encoding plasmid at DIV 7 and then treated with vehicle (CTRL) or LP-211 (LP, 100 nM), alone or in combination with the selective 5-HT7R antagonist SB269970 (SB, 100 nM; dotted bars) for three days (from DIV 10 to DIV 12). For each experimental point, cultures were prepared at least in independent triplicates. The bars represent means ± SEM from randomly selected fields for each cell culture condition (n = 60 for WT and n = 51 for 5-HT7R-KO neurons). Asterisk (*): values significantly different from the respective CTRL by one-way ANOVA followed by Tukey post-hoc test ($p < 0.01$). Hash mark (#): values significantly different from each other by one-way ANOVA followed by Tukey post-hoc test ($p < 0.05$). (B) Representative images of

For Peer Review

WT and 5-HT7-KO cortical neurons treated with vehicle or with LP, SB and LP+SB. White boxes indicate the area of enlargement. Scale bar 20μm.

Fig. 5 Activation of 5-HT7R enhances the formation of synapses in postnatal cultures. (A) Quantification of the co-localization of postsynaptic density protein (PSD-95) and synaptophysin (SYN) in WT striatal primary cultures. The number of co-localized puncta/unit area (arbitrary units) is shown for neurons treated with vehicle (CTRL) or with LP-211 (LP, 100 nM) for three days starting from DIV 10. The bars represent means ± SEM from randomly selected fields for each experimental point. Values represent means ± SEM from randomly selected fields for each cell culture condition (n = 90). Asterisk (*): values significantly different from CTRL by one-way ANOVA followed by Tukey post-hoc test ($p < 0.01$). (B) Representative images of striatal WT neurons transfected with GFP (white) and either untreated (CTRL) or treated with LP followed by immunostaining for synaptophysin (red) and PSD-95 (green) . White boxes in the merged images indicate the magnified area. Magnification shows co-localization of synaptophysin and PSD-95. Scale bar 2 μm.

Fig. 6. Formation of dendritic protrusions induced by 5-HT7R stimulation is mediated by Cdk5 and Cdc42 signaling pathways. (A) Density of dendritic spines (average of the total number of dendritic protrusions/50 μm dendrite) in striatal primary neuronal cultures expressed as percent values of control cells. Cells from WT and 5-HT7R-KO mice were transfected with GFP reporter at DIV 7 and treated at DIV 10 for three days with vehicle (CTRL) or with LP-211 (LP, 100 nM), with or without of roscovitine (ROSCO, 20 μM) or ZCL (50 μM). For each experimental point, cultures were prepared at least in independent triplicates. Values represent means ± SEM from randomly selected fields for each cell culture condition (n = 60 for WT and $15 \leq n \geq 19$ for 5-HT7R-KO neurons). Asterisk (*): values significantly different from CTRL

by one-way ANOVA followed by Tukey post-hoc test ($p < 0.01$). (B) Representative images from WT and 5-HT7R-KO neurons treated with vehicle or LP, ROSCO, LP+ROSCO, ZCL and LP+ZCL. White boxes indicate the area of the enlargement. Scale bar 20µm.

References

Adriani, W., Leo, D., Greco, D., Rea, M., di Porzio, U., Laviola, G. and Perrone-Capano, C. (2006) Methylphenidate administration to adolescent rats determines plastic changes on reward-related behavior and striatal gene expression. *Neuropsychopharmacology,* **31,** 1946-1956.

Bhattacharya, A., Kaphzan, H., Alvarez-Dieppa, A.C., Murphy, J.P., Pierre, P., and Klann, E. (2012). Genetic Removal of p70 S6 Kinase 1 Corrects Molecular, Synaptic, and Behavioral Phenotypes in Fragile X Syndrome Mice. *Neuron,* **76**, 325–337.

Bijata, M., Wlodarczyk, J., Figiel, I. (2015) Dystroglycan controls dendritic morphogenesis of hippocampal neurons in vitro**.** *Frontiers in Cellular Neuroscience,* doi:10.3389/fncel.2015.00199

Cajal, S. R. (1988) Estructura de los centros nerviosos de las aves. *Rev. Trim. Histol. Norm. Patol.,* **1,** 1-10.

Canese, R., Zoratto, F., Altabella, L. et al. (2015) Persistent modification of forebrain networks and metabolism in rats following adolescent exposure to a 5-HT7 receptor agonist. *Psychopharmacology (Berl),* **232,** 75-89.

Chang, K. T., Ro, H., Wang, W. and Min, K. T. (2013) Meeting at the crossroads: common mechanisms in Fragile X and Down syndrome. *Trends in neurosciences,* **36,** 685-694.

Cheng, P. L. and Poo, M. M. (2012) Early events in axon/dendrite polarization. *Annual review of neuroscience,* **35,** 181-201.

Costa, L., Spatuzza, M., D'Antoni, S. et al. (2012) Activation of 5-HT7 serotonin receptors reverses metabotropic glutamate receptor-mediated synaptic plasticity in wild-type and Fmr1 knockout mice, a model of Fragile X syndrome. *Biol Psychiatry,* **72,** 924-933.

Cotecchia, S., Exum, S., Caron, M. G. and Lefkowitz, R. J. (1990) Regions of the alpha 1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function. *Proceedings of the National Academy of Sciences of the United States of America,* **87,** 2896-2900.

Daubert, E. A. and Condron, B. G. (2010) Serotonin: a regulator of neuronal morphology and circuitry. *Trends Neurosci,* **33,** 424-434.

De Camilli, P., Benfenati, F., Valtorta, F. and Greengard, P. (1990) The synapsins. *Annual review of cell biology,* **6,** 433-460.

De Filippis, B., Chiodi, V., Adriani, W., Lacivita, E., Mallozzi, C., Leopoldo, M., Domenici, M. R., Fuso, A. and Laviola, G. (2015) Long-lasting beneficial effects of central serotonin receptor 7 stimulation in female mice modeling Rett syndrome. *Frontiers in behavioral neuroscience,* **9,** 86.

De Paola, V., Arber, S. and Caroni, P. (2003) AMPA receptors regulate dynamic equilibrium of presynaptic terminals in mature hippocampal networks. *Nature neuroscience,* **6,** 491-500.

For Peer Review

Drerup, J. M., Hayashi, K., Cui, H. et al. (2010) Attention-deficit/hyperactivity phenotype in mice lacking the cyclin-dependent kinase 5 cofactor p35. *Biological psychiatry,* **68,** 1163-1171.
Duhr, F., Deleris, P., Raynaud, F. et al. (2014) Cdk5 induces constitutive activation of 5-HT6 receptors to promote neurite growth. *Nature chemical biology,* **10,** 590-597.
Fernandes, D. and Carvalho, A. L. (2016) Mechanisms of homeostatic plasticity in the excitatory synapse. *J Neurochem*.
Fiala, J. C., Spacek, J. and Harris, K. M. (2002) Dendritic spine pathology: cause or consequence of neurological disorders? *Brain research. Brain research reviews,* **39,** 29-54.
Garner, C. C., Nash, J. and Huganir, R. L. (2000) PDZ domains in synapse assembly and signalling. *Trends in cell biology,* **10,** 274-280.
Hagan, J. J., Price, G. W., Jeffrey, P. et al. (2000) Characterization of SB-269970-A, a selective 5-HT(7) receptor antagonist. *Br J Pharmacol,* **130,** 539-548.
Hedlund, P. B., Danielson, P. E., Thomas, E. A., Slanina, K., Carson, M. J. and Sutcliffe, J. G. (2003) No hypothermic response to serotonin in 5-HT7 receptor knockout mice. *Proc Natl Acad Sci U S A,* **100,** 1375-1380.
Hedlund, P. B., Leopoldo, M., Caccia, S., Sarkisyan, G., Fracasso, C., Martelli, G., Lacivita, E., Berardi, F. and Perrone, R. (2010) LP-211 is a brain penetrant selective agonist for the serotonin 5-HT(7) receptor. *Neurosci Lett,* **481,** 12-16.
Hering, H. and Sheng, M. (2001) Dendritic spines: structure, dynamics and regulation. *Nature reviews. Neuroscience,* **2,** 880-888.
Holtmaat, A. and Svoboda, K. (2009) Experience-dependent structural synaptic plasticity in the mammalian brain. *Nature reviews. Neuroscience,* **10,** 647-658.
Hong, S. B., Zalesky, A., Fornito, A. et al. (2014) Connectomic disturbances in attention-deficit/hyperactivity disorder: a whole-brain tractography analysis. *Biological psychiatry,* **76,** 656-663.
Jaffe, A. B. and Hall, A. (2005) Rho GTPases: biochemistry and biology. *Annual review of cell and developmental biology,* **21,** 247-269.
Kawauchi, T. (2014) Cdk5 regulates multiple cellular events in neural development, function and disease. *Development, growth & differentiation,* **56,** 335-348.
Kayser, M. S., Nolt, M. J. and Dalva, M. B. (2008) EphB receptors couple dendritic filopodia motility to synapse formation. *Neuron,* **59,** 56-69.
Kobe, F., Guseva, D., Jensen, T. P. et al. (2012) 5-HT7R/G12 signaling regulates neuronal morphology and function in an age-dependent manner. *J Neurosci,* **32,** 2915-2930.
Kvachnina, E., Liu, G., Dityatev, A. et al. (2005) 5-HT7 receptor is coupled to G alpha subunits of heterotrimeric G12-protein to regulate gene transcription and neuronal morphology. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* **25,** 7821-7830.
Lai, K. O. and Ip, N. Y. (2013) Structural plasticity of dendritic spines: the underlying mechanisms and its dysregulation in brain disorders. *Biochimica et biophysica acta,* **1832,** 2257-2263.
Lefebvre, J. L., Sanes, J. R. and Kay, J. N. (2015) Development of dendritic form and function. *Annual review of cell and developmental biology,* **31,** 741-777.
Lei, W., Omotade, O. F., Myers, K. R. and Zheng, J. Q. (2016) Actin cytoskeleton in dendritic spine development and plasticity. *Current opinion in neurobiology,* **39,** 86-92.
Leo, D., Adriani, W., Cavaliere, C. et al. (2009) Methylphenidate to adolescent rats drives enduring changes of accumbal Htr7 expression: implications for impulsive behavior and neuronal morphology. *Genes, brain, and behavior,* **8,** 356-368.

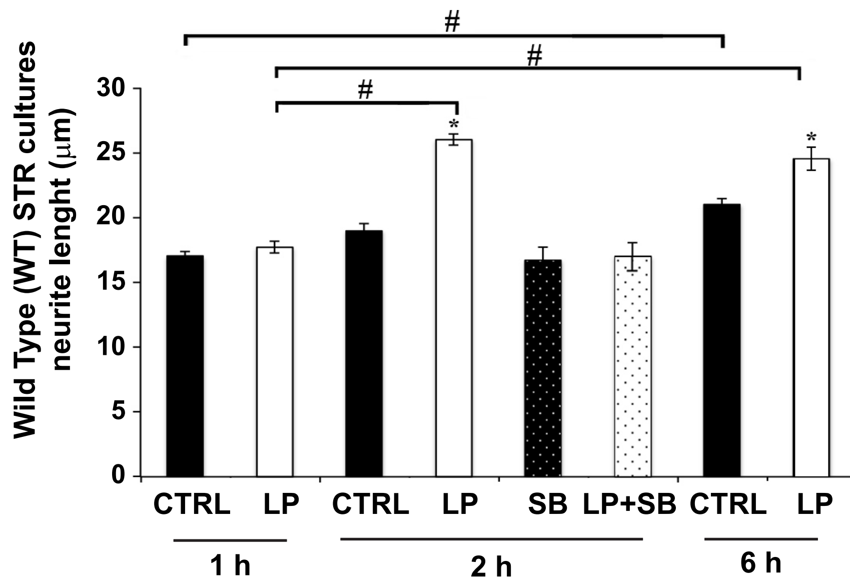
For Peer Review

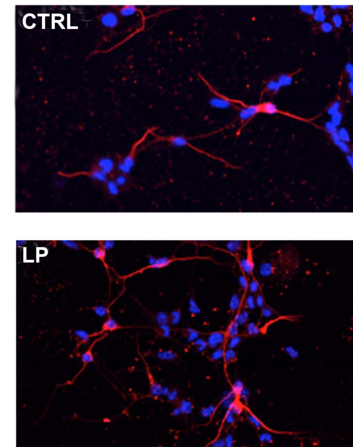
Leopoldo, M., Lacivita, E., Berardi, F., Perrone, R. and Hedlund, P. B. (2011) Serotonin 5-HT7 receptor agents: Structure-activity relationships and potential therapeutic applications in central nervous system disorders. *Pharmacol Ther,* **129,** 120-148.
Lesch, K. P. and Waider, J. (2012) Serotonin in the modulation of neural plasticity and networks: implications for neurodevelopmental disorders. *Neuron,* **76,** 175-191.
Matthys, A., Haegeman, G., Van Craenenbroeck, K. and Vanhoenacker, P. (2011) Role of the 5-HT7 receptor in the central nervous system: from current status to future perspectives. *Mol Neurobiol,* **43,** 228-253.
Mattila, P. K. and Lappalainen, P. (2008) Filopodia: molecular architecture and cellular functions. *Nature reviews. Molecular cell biology,* **9,** 446-454.
Mishiba, T., Tanaka, M., Mita, N., He, X., Sasamoto, K., Itohara, S. and Ohshima, T. (2014) Cdk5/p35 functions as a crucial regulator of spatial learning and memory. *Molecular brain,* **7,** 82.
Mita, N., He, X., Sasamoto, K., Mishiba, T. and Ohshima, T. (2016) Cyclin-Dependent Kinase 5 Regulates Dendritic Spine Formation and Maintenance of Cortical Neuron in the Mouse Brain. *Cerebral cortex,* **26,** 967-976.
Morita, T., McClain, S. P., Batia, L. M. et al. (2015) HTR7 Mediates Serotonergic Acute and Chronic Itch. *Neuron,* **87,** 124-138.
Naumenko, V. S. P., N.K.; Lacivita, E.; Leopoldo, M.; Ponimaskin, E.G. (2014) Interplay between Serotonin 5-HT1A and 5-HT7 Receptors in Depressive Disorders. *CNS Neuroscience & Therapeutics*.
Ponimaskin, E., Voyno-Yasenetskaya, T., Richter, D. W., Schachner, M. and Dityatev, A. (2007) Morphogenic signaling in neurons via neurotransmitter receptors and small GTPases. *Molecular neurobiology,* **35,** 278-287.
Renner, U., Zeug, A., Woehler, A., Niebert, M., Dityatev, A., Dityateva, G., Gorinski, N., Guseva, D., Abdel-Galil, D., Fröhlich, M., Döring, F., Wischmeyer, E., Richter, D.W., Neher, E., Ponimaskin, E.G. (2012) Heterodimerization of serotonin receptors 5-HT1A and 5-HT7 differentially regulates receptor signalling and trafficking. *Journal of Cell Science*, **125,** 2486-2499
Roberts, A. J. and Hedlund, P. B. (2012) The 5-HT(7) receptor in learning and memory. *Hippocampus,* **22,** 762-771.
Rosenbaum, D. M., Rasmussen, S. G. and Kobilka, B. K. (2009) The structure and function of G-protein-coupled receptors. *Nature,* **459,** 356-363.
Sala, C. and Segal, M. (2014) Dendritic spines: the locus of structural and functional plasticity. *Physiological reviews,* **94,** 141-188.
Santello, M. and Nevian, T. (2015) Dysfunction of cortical dendritic integration in neuropathic pain reversed by serotoninergic neuromodulation. *Neuron,* **86,** 233-246.
Seifert, R. and Wenzel-Seifert, K. (2002) Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. *Naunyn-Schmiedeberg's archives of pharmacology,* **366,** 381-416.
Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. 3, 1101-1108.
Speranza, L., Chambery, A., Di Domenico, M. et al. (2013) The serotonin receptor 7 promotes neurite outgrowth via ERK and Cdk5 signaling pathways. *Neuropharmacology,* **67,** 155-167.
Speranza, L., Giuliano, T., Volpicelli, F. et al. (2015) Activation of 5-HT7 receptor stimulates neurite elongation through mTOR, Cdc42 and actin filaments dynamics. *Frontiers in behavioral neuroscience,* **9,** 62.

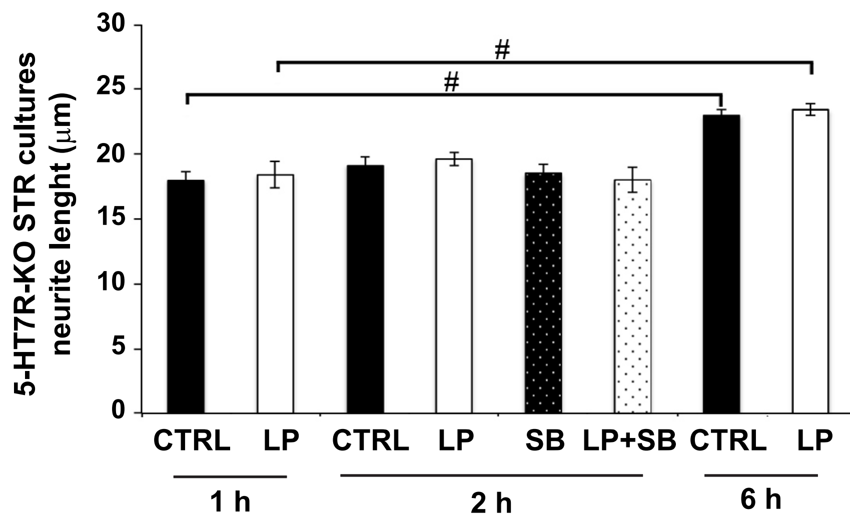
Takano, T., Xu, C., Funahashi, Y., Namba, T. and Kaibuchi, K. (2015) Neuronal polarization. *Development,* **142,** 2088-2093.
Tau, G. Z. and Peterson, B. S. (2010) Normal development of brain circuits. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology,* **35,** 147-168.
Vizuete, M. L., Venero, J. L., Traiffort, E., Vargas, C., Machado, A. and Cano, J. (1997) Expression of 5-HT7 receptor mRNA in rat brain during postnatal development. *Neurosci Lett,* **227,** 53-56.
Volpicelli, F., Speranza, L., di Porzio, U., Crispino, M. and Perrone-Capano, C. (2014) The serotonin receptor 7 and the structural plasticity of brain circuits. *Frontiers in behavioral neuroscience,* **8,** 318.
Wirth, A., Holst, K. and Ponimaskin, E. (2016) How serotonin receptors regulate morphogenic signalling in neurons. *Progress in neurobiology*.
Woolfrey, K. M. and Srivastava, D. P. (2016) Control of Dendritic Spine Morphological and Functional Plasticity by Small GTPases. *Neural plasticity,* **2016,** 3025948.
Yuste, R. (2011) Dendritic spines and distributed circuits. *Neuron,* **71,** 772-781.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60







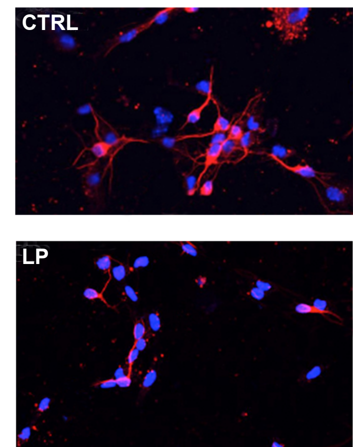


Figure 1

A

Wild Type (WT) CTX cultures neurite lenght (μm)

30
25
20
15
10
5
0

#
#
*
*

CTRL LP CTRL LP SB LP+SB CTRL LP

1 h 2 h 6 h

CTX WT

CTRL

LP

B

5-HT7R-KO CTX cultures neurite lenght (μm)

30
25
20
15
10
5
0

#
#

CTRL LP CTRL LP SB LP+SB CTRL LP

1 h 2 h 6 h

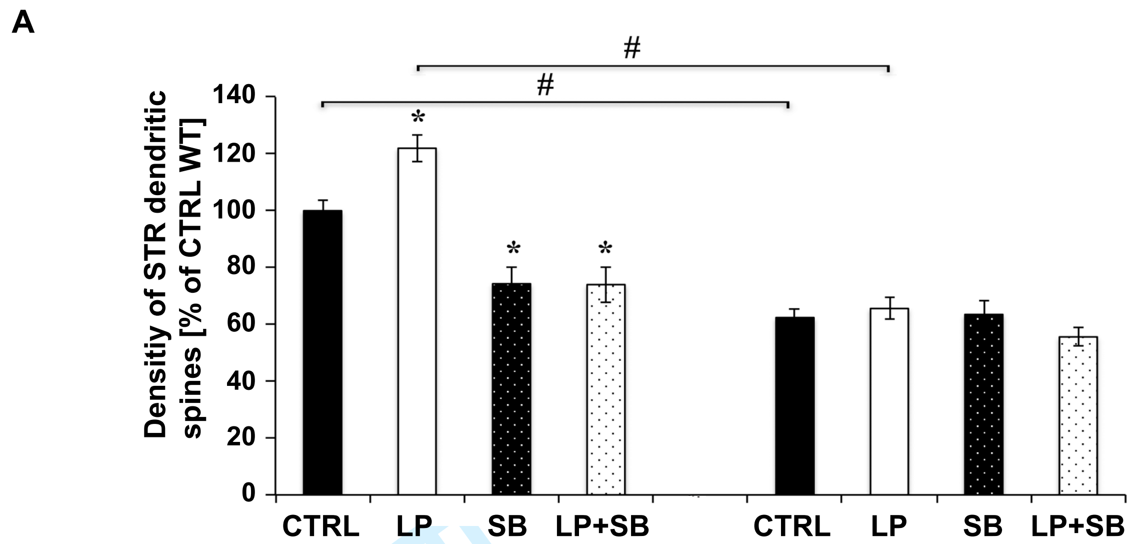
CTX KO

CTRL

LP

Figure 2

A



B

WT

KO

CTRL

SB

CTRL

SB

LP

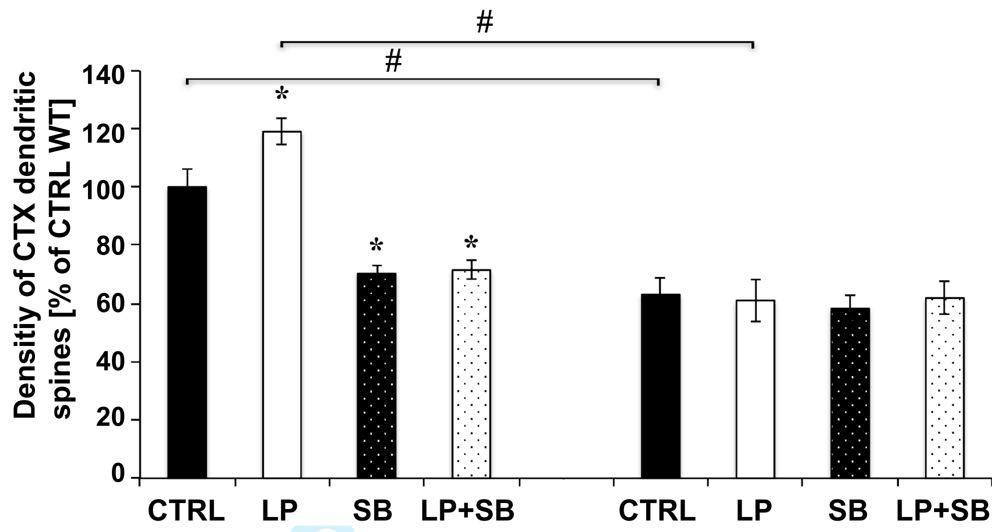
LP + SB

LP

LP + SB

Figure 3

A



B

WT

KO

CTRL

SB

CTRL

SB

LP

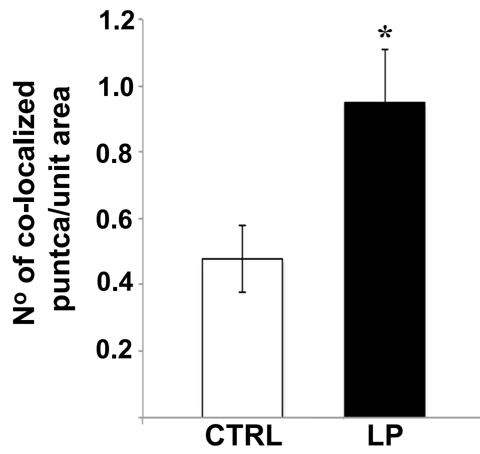
LP + SB

LP

LP + SB

Figure 4

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60



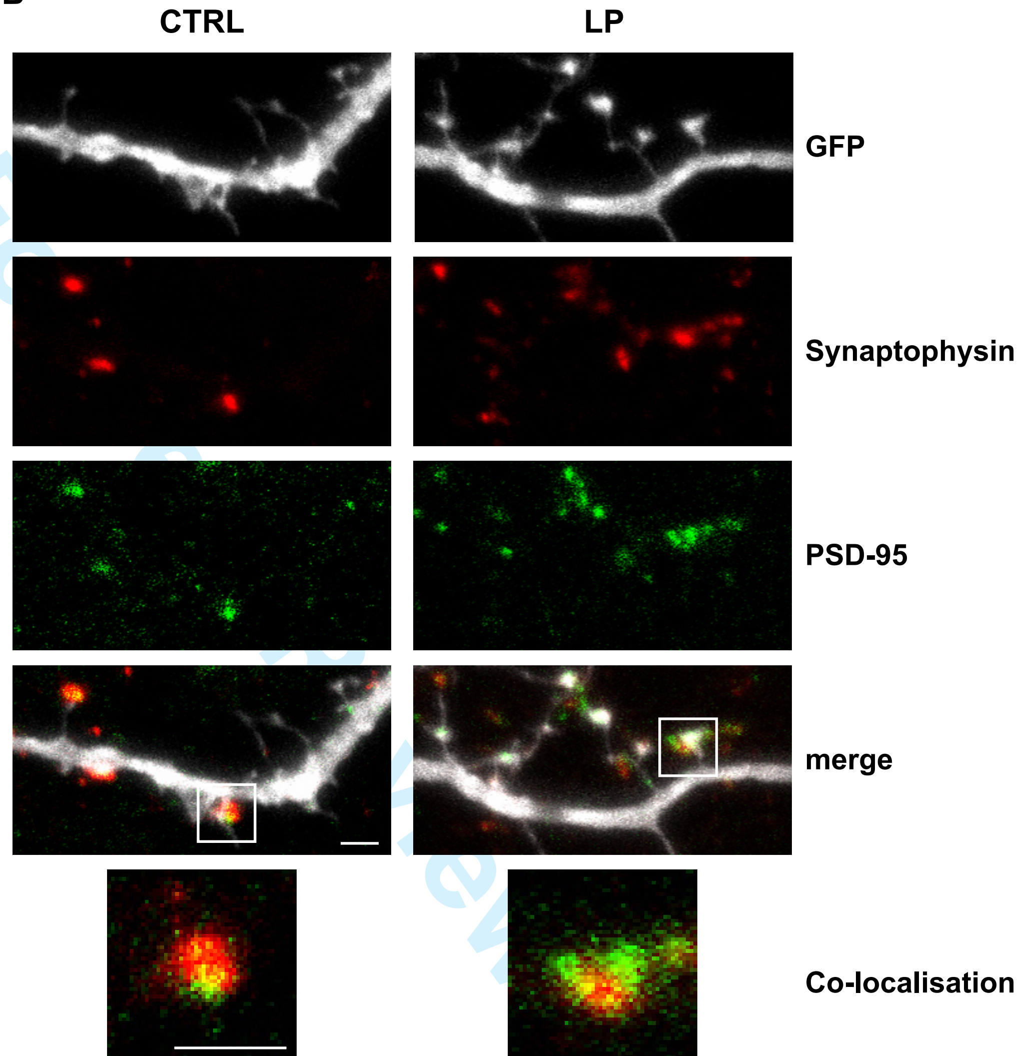
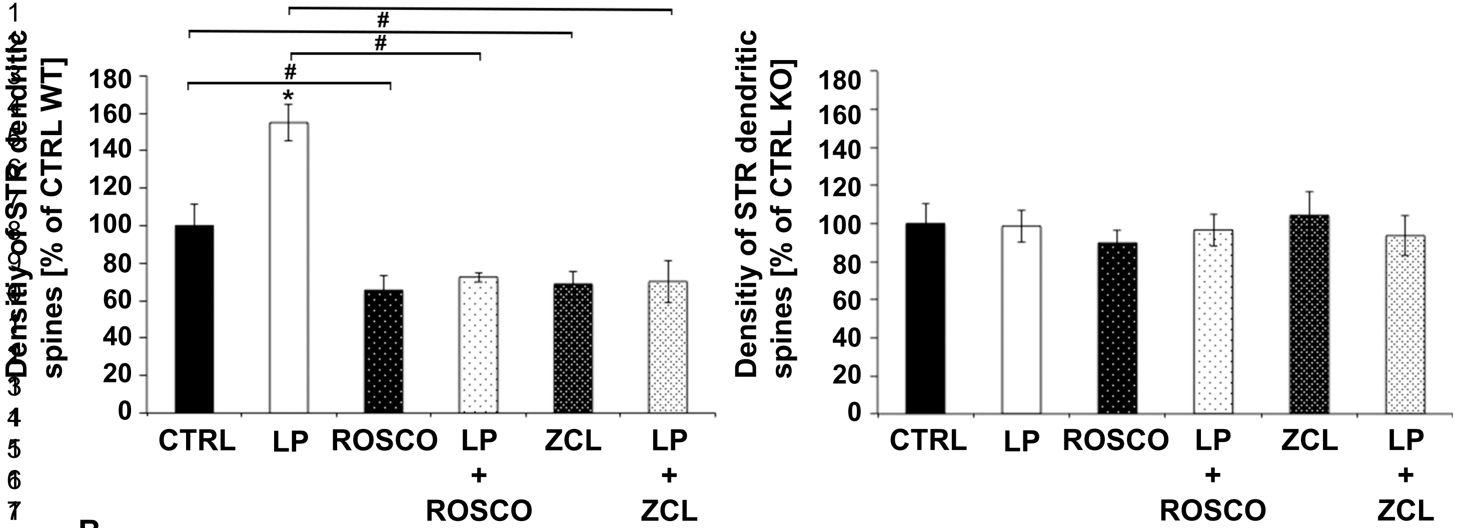


Figure 5



B

WT

KO

CTRL
LP
CTRL
LP

ROSCO
LP + ROSCO
ROSCO
LP + ROSCO

ZCL
LP + ZCL
ZCL
LP + ZCL

Figure 6

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Supplementary data

Fig. S1 Primary branching of neuronal cultures from wild-type (WT) and 5-HT7R-KO mice. Primary branching (evaluated as neurites originating directly from soma) was evaluated on striatal (**A**) and cortical **(B**) neurons obtained from WT and 5-HT7R-KO mice. Cells were transfected with GFP-encoding plasmid and treated with vehicle (CTRL) or LP-211 (LP, 100 nM) for three days (from DIV 10 to DIV 12). Values represent means ± SEM from randomly selected fields for each cell culture condition (n = 20 for WT and n = 15 for KO). Hash mark (#): values significantly different from each other by one-way ANOVA followed by Tukey post-hoc test ($p < 0.05$).

Fig. S2 Expression levels of 5-HT7R assessed by RT-PCR do not vary in neuronal cultures between 4 and 12 days *in vitro* (DIV). The bars represent the 5-HT7R mRNA levels determined by quantitative RT-PCR and normalized to housekeeping gene HPRT (means ± SEM; n = 6) in striatal (STR, black) and cortical (CTX, white) neurons at DIV 4 and at DIV 12.

Fig. S3 Cdc42 activation is induced by 5-HT7R stimulation. Western blot analysis of active Cdc42 (Cdc42-GTP) and total Cdc42 in cortical WT and 5-HT7R-KO neurons treated with vehicle (CTRL), LP-211 (LP, 100 nM), alone or in combination with the selective 5-HT7R antagonist SB269970 (SB, 100 nM; dotted bars) for three days (from DIV 10 to DIV 12) after GST-PAK-PBD pull-down. Representative Western blot out of four independent experiments is shown. GAPDH serves as loading control for cell lysate input.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

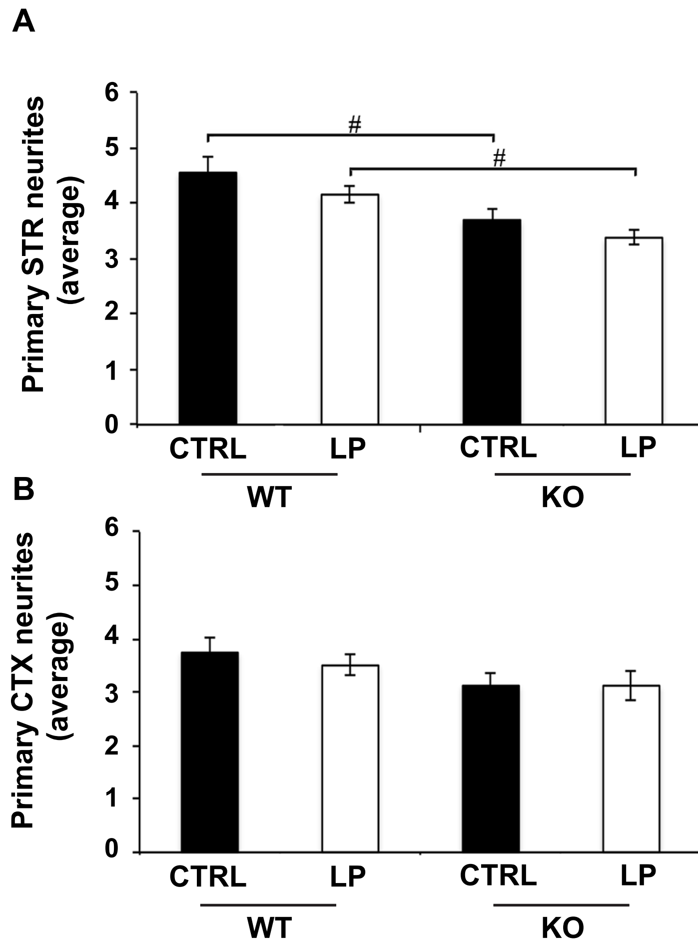
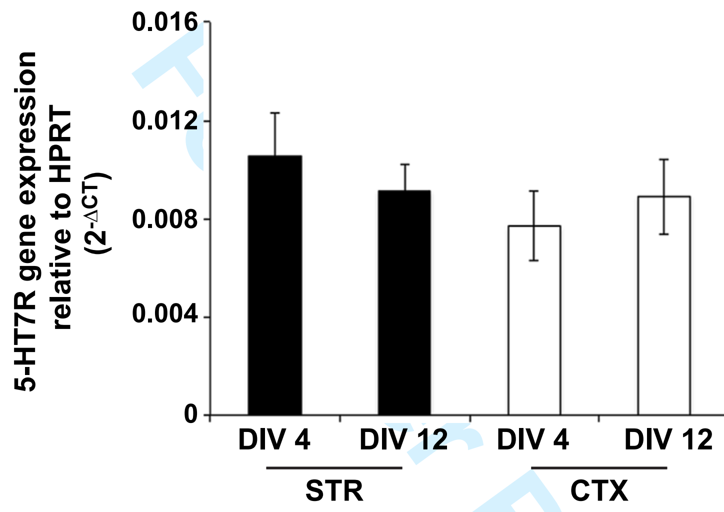


Figure S1

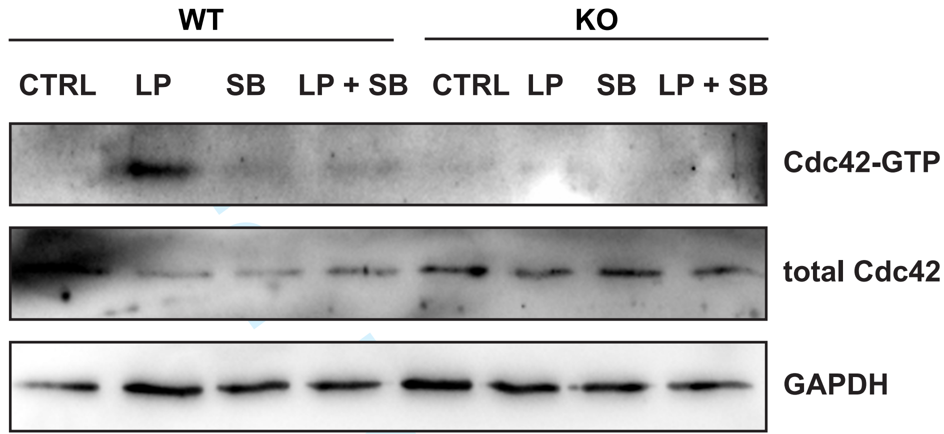
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



For Peer Review

Figure S2

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



For Peer Review

Figure S3