Neuropharmacology 67 (2013) 155–167

Contents lists available at SciVerse ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

The serotonin receptor 7 promotes neurite outgrowth via ERK and Cdk5 signaling pathways

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ARTICLE INFO

Article history:
Received 20 June 2012
Received in revised form
3 August 2012
Accepted 9 October 2012

Keywords:
5-HTR7
Cytoskeletal proteins
MAP1B
Neurite outgrowth
Neuronal primary cultures

ABSTRACT

Serotonergic neurotransmission is mediated by at least 14 subtypes of 5-HT receptors. Among these, the CNS serotonin receptor 7 (5-HTR7) is involved in diverse physiological processes. Here we show that treatment of murine striatal and cortical neuronal cultures with 5-HTR7 agonists (8-OH-DPAT and LP-211) significantly enhances neurite outgrowth. This effect is abolished by the selective 5-HTR7 antagonist SB-269970, by the ERK inhibitor U0126, by the cyclin-dependent kinase 5 (Cdk5) inhibitor roscovitine, as well as by cycloheximide, an inhibitor of protein synthesis. These data indicate that 5-HTR7 activation stimulates extensive neurite elongation in CNS primary cultures, subserved by ERK and Cdk5 activation, and de novo protein synthesis.

Two-dimensional (2D) gel electrophoresis coupled to Western blot analyses reveals both qualitative and quantitative expression changes in selected cytoskeletal proteins, following treatment of striatal primary cultures with LP-211. In particular, the 34 kDa isoform of MAP1B is selectively expressed in stimulated cultures, consistent with a role of this protein in tubulin polymerization and neurite elongation.

In summary, our results show that agonist-dependent activation of the endogenous 5-HTR7 in CNS neuronal primary cultures stimulates ERK- and Cdk5-dependent neurite outgrowth, sustained by modifications of cytoskeletal proteins. These data support the hypothesis that the 5-HTR7 might play a crucial role in shaping neuronal morphology and behaviorally relevant neuronal networks, paving the way to new approaches able to modulate CNS connectivity.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) action in the Central Nervous System is implicated in the regulation of a variety of physiological and pathological processes. Mammalian brain contains extensive serotonergic projections that exert their effects through at least 14 different subtypes of 5-HT receptors (5-HTRs), each encoded by a distinct gene (Barnes and Sharp, 1999; Pytliak et al., 2011).

In addition to its role in neurotransmission, 5-HT regulates many processes during neural development, including modulation of neuronal morphology and survival (Ponimaskin et al., 2007). Recent data suggest that the structural organization of behaviorally relevant neuronal networks during sensitive developmental stages may be modulated by the activation of the serotonin receptor 7 (5-HTR7), which could have a prominent role in regulating the neuronal cytoarchitecture. We have previously shown that in adolescent rodents 5-HTR7 plays a major role in the modulation of self-control behavior, and may subserve the persistent structural rearrangements of the brain reward pathways occurring during postnatal development, following chronic methyphenidate

Abbreviations: 5-HT, 5-hydroxytryptamine; 5-HTR7, serotonin receptor 7; ADHD, attention deficit hyperactivity disorder; Cdk5, cyclin-dependent kinase 5; CHX, cycloheximide; CTX, cortex; E, embryonic age; ERK, extracellular signal-regulated kinases 1/2; GABA, gamma-aminobutyric acid; GAD2, glutamate decarboxylase 2; HPRT, hypoxanthine phosphoribosyltransferase; MAP, microtubule-associated protein; NFL, neurofilament light; NFM, neurofilament medium; STR, striatum.

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http://dx.doi.org/10.1016/j.neuropharm.2012.10.026

exposure (Leo et al., 2009). Moreover, it has been recently shown that 5-HTR7 modulates hippocampal neuronal morphology, excitability and plasticity in an age-dependent manner (Kobe et al., 2012).

The 5-HTR7 is expressed both in the central and peripheral nervous systems, and has been cloned from several animal species including human (Hedlund and Sutcliffe, 2004). In adult human and rodent brains, the highest densities of 5-HTR7 have been observed in hypothalamus, thalamus, hippocampus, cortex, amygdala and striatal complex (Cifariello et al., 2008; Leo et al., 2009). Recent data suggest that *in vivo* the 5-HTR7s exist as homodimers or heterodimers with the 5-HTR1A and that these oligomers can affect the biological function of the monomers involved in the interaction (Renner et al., 2012; Teitler and Klein, 2012).

Pharmacological, genetic and behavioral approaches have clarified the physiological role of the 5-HTR7 in homeostasis maintenance, acting on circadian rhythms, body temperature, endocrine regulation, and nociception (Hedlund et al., 2003, 2004; Faure et al., 2006; Matthys et al., 2011; Thomas et al., 2003). Moreover, strong evidence supports its involvement in specific aspects of hippocampus-dependent contextual learning and memory processing (Gasbarri et al., 2008; Eriksson et al., 2008; Sarkisyan and Hedlund, 2009).

An important role of the 5-HTR7 in mood regulation has been suggested by the observations that several antipsychotics and antidepressants have high affinity for this receptor (Mullins et al., 1999; Roth et al., 1994), and that modulation of this receptor might produce antidepressant and antianxiety effects (Bonaventure et al., 2007; Mnie-Filali et al., 2007, 2011; Nandam et al., 2007). In addition, 5-HTR7 knock-out mice exhibit a phenotype similar to antidepressant treated mice (Hedlund et al., 2005; Guscott et al., 2005). All together, these data strengthen the hypothesis that 5-HTR7 mediates key aspects of serotonergic transmission in mood regulation.

Other pathological processes of the CNS involving dysregulation of the 5-HTR7 include anxiety, schizophrenia, epilepsy, and migraine (Hedlund, 2009; Matthys et al., 2011).

The 5-HTR7 is a seven-transmembrane G-protein-coupled receptor, positively linked to adenylate cyclase through the stimulatory Gs protein. Its prominent downstream effectors are protein kinase A (PKA) and the extracellular signal-regulated kinases1/2 (ERK, Errico et al., 2001). An additional intracellular signaling pathway activated by the 5- HTR7 involves the Gα-12 subunit of heterotrimeric G-protein, which leads to stimulation of small GTPases, involved in cytoskeletal rearrangements (Kvachnina et al., 2005). In accordance with these observations, agonist-dependent activation of 5-HTR7/G12 signaling induces filopodia formation in 5-HTR7 transfected NIH3T3 cells, and increased neurite length and synaptogenesis in mouse hippocampal neurons (Kobe et al., 2012).

In this work we have used embryonic neuronal primary cultures from areas of rat and mouse brain reward circuits (i.e. striatum complex and cortex) to show that the stimulation of 5-HTR7 regulates neurite outgrowth. We have used a commercially available 5-HTR7 promiscuous agonist (8-OH-DPAT), and a novel highly potent and selective 5-HTR7 agonist, LP-211 (Hedlund et al., 2010). The specific agonistic action of LP-211 has been recently confirmed *in vivo* by comparing its ability to induce hypothermia in control mice ($5\text{-HT}_7^{+/+}$) and not in their littermates lacking 5-HTR7 ($5\text{-HT}_7^{-/-}$; Hedlund et al., 2010). Moreover, we have identified intracellular signaling kinases required for the 5-HTR7-dependent neurite elongation. Finally we show qualitative and quantitative expression changes in selected cytoskeletal proteins, occurring in response to agonist-dependent activation of 5-HTR7.

2. Materials and methods

2.1. Neuronal primary cultures

Timed pregnant Sprague-Dawley rats (Charles River, Italy) or C57 BL/6 mice were housed, cared for and sacrificed in accordance with the recommendations of the European Commission. The embryonic age (E) was determined by the date of insemination (i.e. the appearance of vaginal plug was considered as day E0). About 15–20 embryos from different dams were pooled (at E16 for rats, and at E15 for mice) and were used for preparation of the monolayer cultures. The striatum (STR) and cortex (CTX) were quickly dissected from embryos, under a stereoscope in sterile conditions, and placed in PBS without calcium and magnesium, supplemented with 33 mM glucose. Cells were dissociated from embryonic STR or CTX, and cultured as previously described (di Porzio et al., 1980). Briefly, the dissected areas were enzymatically dissociated by incubation for 30 min (min) at 37 °C in a papain solution (Warthington, 20 U/ml) in Earle's balance salts containing 1 mM EDTA, 1 mM cysteine and 0.01% pancreatic DNAse. After addition of 1 mg/ml of bovine serum albumin (Sigma-Aldrich) and 1 mg/ml ovomucoid (Sigma-Aldrich) the cells suspensions were centrifuged 5 min at 800 g, resuspended in plating medium and counted (Fiszman et al., 1991). For the viable count, cell suspension was diluted 1:1 with 0.1% trypan blue dye and loaded into a disposable cell counting chamber-slide. Cell concentration was determined on the basis of the total cell count, the dilution factor and the trypan blue dye exclusion.

Dissociated cells were plated at a density of $1.5 \times 10^5/\text{cm}^2$ in 2 cm^2 Lab-Tek chamber slides (Nunc) for morphological analyses, and at a density of $3 \times 10^5/\text{cm}^2$ in 9,5 cm^2 cell culture dishes (Corning) for RNA purification and Western blot analyses. Both chamber slides and cell culture dishes were coated with 15 μg/ml of poly-D-Lysine (Sigma-Aldrich).

Cells were grown in serum-free Neurobasal medium (Invitrogen), supplemented with B27 (Invitrogen), 2 mM L-glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml). Cultures were maintained at 37 °C in a humidified incubator for 3 days before experimental manipulation. For each experimental point, cultures were prepared at least in independent triplicates, and experimental points were repeated using independent cell preparations.

2.2. Drugs and reagents

The cell cultures were treated with 100 nM of the 5-HTR (1a/7) agonist 8-OH-DPAT (Tocris), 100 nM of the selective 5-HTR agonist LP-211 (gift of M. Leopoldo, University of Bari, Italy), 100 nM of the HTR7 antagonist SB-269970 (Tocris), or with a combination of these drugs. Pretreatment of cells with the 5-HTR1a antagonist, WAY 100635 (Tocris; 30 nM) was performed for 30 min (Corradetti et al., 1998). Pretreatment of the cells with the ERK1/2 inhibitor U0126 was performed at the final concentration of 10 μM, for 30 min, as recommended by manufacturer (Cell Signalling). Pretreatment of the cells with cycloheximide (Sigma-Aldrich), an inhibitor of eukaryotic protein synthesis, was performed at the final concentration of 50 μM, for 30 min. Roscovitine (Sigma-Aldrich), a Cdk5 inhibitor, was used at the final concentration of 20 μM. Drugs were added to cultures 72 h (h) after cell plating and incubated for appropriate time.

2.3. Morphological characterization and measurement of neurite outgrowth

For immunofluorescence analysis, cells in culture were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), for