FURTHER UNDERSTANDING OF SEROTONIN 7 RECEPTORS' NEURO-PSYCHO-PHARMACOLOGY

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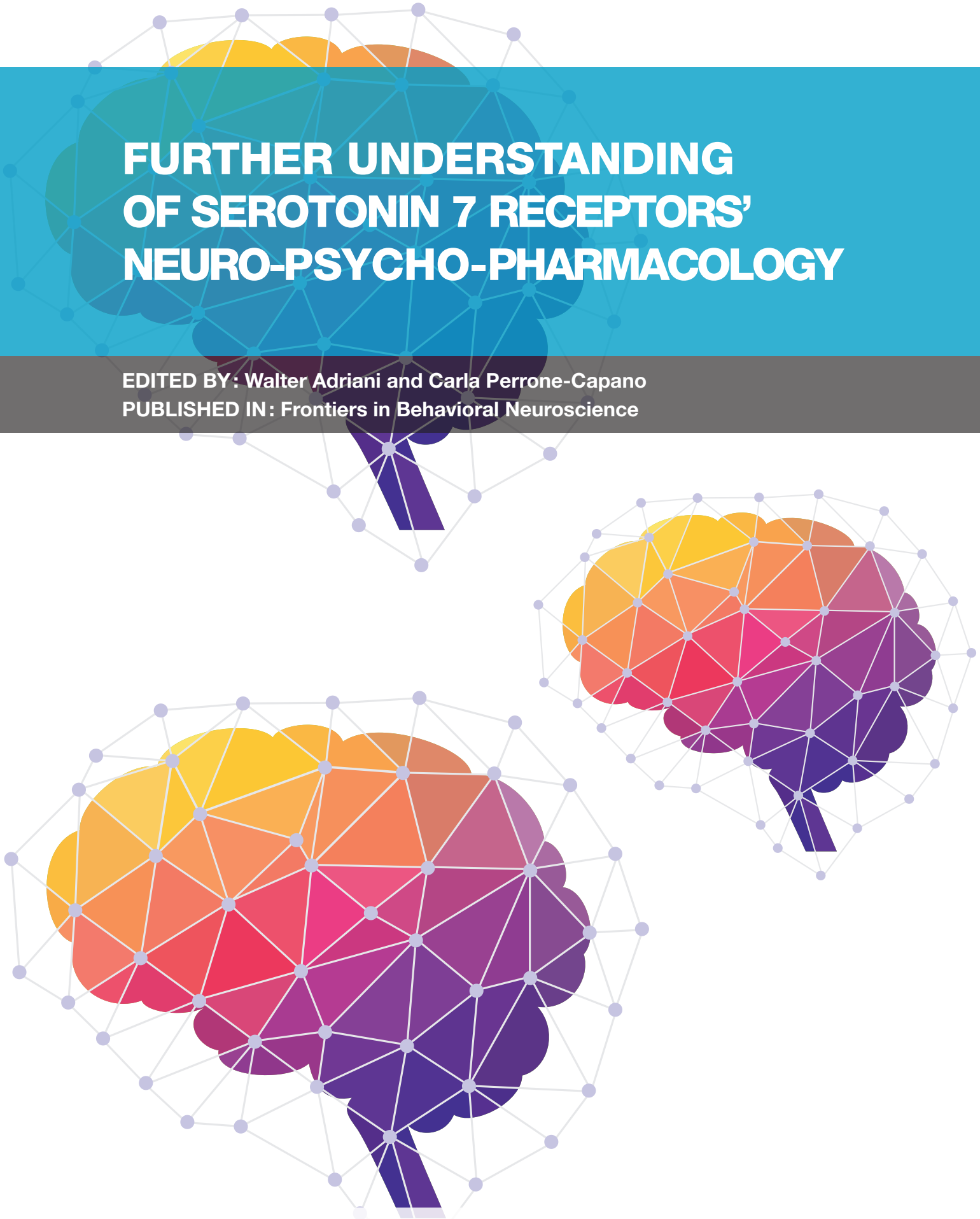

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FURTHER UNDERSTANDING OF SEROTONIN 7 RECEPTORS' NEURO-PSYCHO-PHARMACOLOGY

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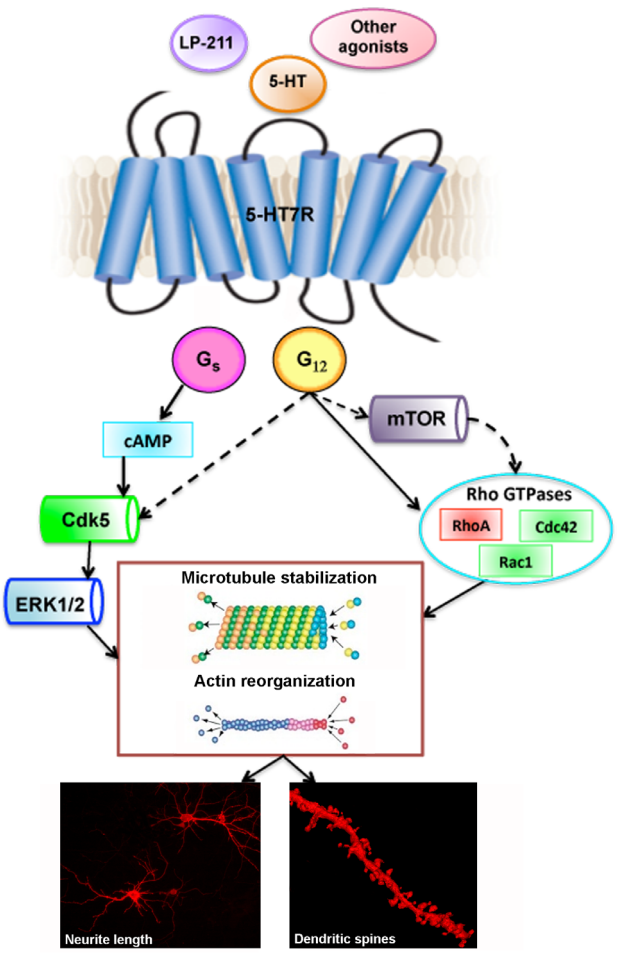

Schematic drawing of the 5-HT7 receptor and its signaling pathways involved in remodeling of neuronal morphology. Established and putative patways are drawn as full and dashed lines, respectively. Lower panels are photomicrographs from DiI-labeled striatal neurons in culture (left panel), and striatal

slices from brain adult mice (right panel). Abbreviations: 5-HT: 5-hydroxytryptamine (serotonin); 5-HT7R: serotonin receptor 7; LP-211: selective 5-HT7R agonist; Cdk5: cyclin-dependent kinase 5; ERK 1/2: extracellular signal-regulated kinases 1/2; mTOR: mammalian target of rapamycin.

Image taken from: 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. Front. Behav. Neurosci. 8:318. doi: 10.3389/fnbeh.2014.00318

Within the CNS and in the periphery, serotonin (5-HT) participates in a number of functions including cognition, mood, sleep-wake rhythms, intestinal inflammation. 5-HT receptors can be classified into at least seven classes, designated 5-HT1 to 5-HT7. Since its identification, the 5-HT7 receptor has been the subject of intense research efforts, driven by its presence in functionally-relevant brain regions and in the gut. The availability of selective agonists and antagonists, in combination with genetically-modified mice lacking 5-HT7 receptors, has allowed so far a better understanding about the patho-physiological roles of this receptor. This Topic will review the state-of-the-art from studies conducted in laboratory (alive animals, tissues, cells) on this respect: 1) Emerging preclinical evidence supports a role for the 5-HT7 receptor in depression, since its pharmacological blockade or genetic inactivation induce an antidepressant-like behavioural profile. 2) In addition, agonists and/or antagonists of 5-HT7 receptors may improve memory or reverse amnesia, having pro-mnesic and/or anti-amnesic effects, with therapy potential in disease-related and/or age-related cognitive impairment. 3) When adolescent rats are treated with a 5-HT7 agonist, analysis at adulthood shows an improved exploratory motivation / attentional skill as well as increased strength of connectivity among components of a forebrain "limbic" loop. 4)In vitro and in vivo studies indicate that 5-HT7R modulates the neuronal morphology, excitability and plasticity, hence contributing to the establishment of brain connectivity during embryonic and early postnatal life.The role of neuro-plasticity and the links to neuro-inflammatory processes will also be addressed. Therapeutic potential of the beneficial effects triggered by 5-HT7 stimulation warrant future research.

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Identification of essential residues for binding and activation in the human 5-HT$_{7(a)}$ serotonin receptor by molecular modeling and site-directed mutagenesis

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The human 5-HT$_7$ receptor is expressed in both the central nervous system and peripheral tissues and is a potential drug target in behavioral and psychiatric disorders. We examined molecular determinants of ligand binding and G protein activation by the human 5-HT$_{7(a)}$ receptor. The role of several key residues in the 7th transmembrane domain (TMD) and helix 8 were elucidated combining *in silico* and experimental mutagenesis. Several single and two double point mutations of the 5-HT$_{7(a)}$ wild type receptor were made (W7.33V, E7.35T, E7.35R, E7.35D, E7.35A, R7.36V, Y7.43A, Y7.43F, Y7.43T, R8.52D, D8.53K; E7.35T-R7.36V, R8.52D-D8.53K), and their effects upon ligand binding were assessed by radioligand binding using a potent agonist (5-CT) and a potent antagonist (SB269970). In addition, the ability of the mutated 5-HT$_{7(a)}$ receptors to activate G protein after 5-HT-stimulation was determined through activation of adenylyl cyclase. *In silico* investigation on mutated receptors substantiated the predicted importance of TM7 and showed critical roles of residues E7.35, W7.33, R7.36 and Y7.43 in agonist and antagonist binding and conformational changes of receptor structure affecting adenylyl cyclase activation. Experimental data showed that mutants E7.35T and E7.35R were incapable of ligand binding and adenylyl cyclase activation, consistent with a requirement for a negatively charged residue at this position. The mutant R8.52D was unable to activate adenylyl cyclase, despite unaffected ligand binding, consistent with the R8.52 residue playing an important role in the receptor-G protein interface. The mutants Y7.43A and Y7.43T displayed reduced agonist binding and AC agonist potency, not seen in Y7.43F, consistent with a requirement for an aromatic residue at this position. Knowledge of the molecular interactions important in h5-HT$_7$ receptor ligand binding and G protein

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activation will aid the design of selective h5-HT$_7$ receptor ligands for potential pharmacological use.

Keywords: homology modeling, mutagenesis, molecular dynamics, docking, G protein, adenylyl cyclase

Introduction

The 5-HT$_7$ receptor is a seven-transmembrane spanning receptor, coupled primarily to the stimulatory G protein (G_s). It is found in multiple organ systems, such as the cardiovascular system, CNS and digestive tract. In the central nervous system it has been proposed to play a role in the action of antipsychotics and antidepressants and it seems to be involved in regulating circadian rhythms and thermoregulation, learning and memory, as well as rapid eye movement (REM) sleep via the modulation of suprachiasmatic nucleus neurons (Gellynck et al., 2013). 5-HT$_7$ receptor agonists have been suggested in treatment of dysfunctional memory in age-related decline and Alzheimer's disease (Meneses, 2014), as well as treatment of pain, migraine, schizophrenia, anxiety and cognitive disturbances (Gellynck et al., 2013; Gasbarri and Pompili, 2014). A possible involvement in regulation of mood suggests that 5-HT$_7$ is a potential target for the treatment of depression (Gellynck et al., 2013). In the periphery the 5-HT$_7$ receptor is found primarily in the smooth muscle cells of blood vessels (Ullmer et al., 1995), and in the gastrointestinal tract, where it mediates relaxation of the ileum and stomach (Prins et al., 1999), and was recently shown to be important in inflammation (Guseva et al., 2014).

Three human 5-HT$_7$ receptor splice variants (h5-HT$_{7(a)}$, h5-HT$_{7(b)}$, h5-HT$_{7(d)}$) have been identified that are structurally identical except in their predicted intracellular carboxyl terminal (C-terminal) tail. They have indistinguishable pharmacological properties and similar abilities to stimulate adenylyl cyclase, indicating that the C-terminal tail does not influence ligand binding or G-protein coupling (Krobert et al., 2001). To facilitate development of selective drugs targeting the h5-HT$_7$ receptor, it is necessary to understand the molecular interactions involved in ligand binding to the receptor. Several studies have focused on the molecular interaction of endogenous serotonin with different 5-HT receptor subtypes. Molecular requirements for serotonin binding to its receptor include electrostatic interaction between the receptor and the amino group of the ligand, one hydrogen bond between donor-acceptor site of the receptor and the hydroxyl group of serotonin and finally van der Waals interactions.

Mutagenesis studies of serotonin receptors (Ho et al., 1992; Wang et al., 1993; Boess et al., 1998; Mialet et al., 2000) suggested that the serotonin amino group makes an electrostatic interaction with the carboxylate of the highly conserved aspartate D3.32. This interaction is usually found in biogenic amine receptors, generally involved in binding of agonists/antagonists and in receptor activation. And there is evidence that serotonin hydroxyl groups interact with a donor-acceptor hydrogen bond residue present in transmembrane domain (TMD) V. These interactions seem relevant to only some serotonin receptor subtypes including 5-HT$_{1A}$, 5-HT$_2$, 5-HT$_6$ and 5-HT$_4$ (Mialet et al., 2000). The role of D3.32 was not relevant for antagonist interactions at the 5-HT$_{1A}$ receptor and the role of the hydrogen bond donor residue of the TMDV was not unambiguously identified in either 5-HT$_{1A}$ or 5-HT$_{2B}$ receptors (Wang et al., 2013). These anomalies indicate that the role of individual residues in molecular receptor-ligand interactions vary among different serotonin receptor subtypes.

To determine the critical molecular interactions that mediate ligand binding in the h5-HT$_7$ receptor we used an experimental and *in silico* site-directed mutagenesis approach. Using homology modeling of the β_2-adrenergic receptor (pdb: 2RH1; Cherezov et al., 2007), a 3D model of the h5-HT$_7$ receptor was built and stabilized by large scale simulation in membrane bilayers. We focused on several specific amino acids, located in the 7th TMD, on the basis of preliminary modeling considerations, as discussed in the results section. In order to verify the predicted interactions, several h5-HT$_{7(a)}$ receptor mutants were generated, expressed and assessed for ligand binding as well as agonist and antagonist effects on adenylyl cyclase activity. Our results indicated that residues E7.35, R7.36 and Y7.43 are critical for ligand binding, while residue R8.52 plays a key role in G protein activation.

Methods

Mutation Strategy and Mutagenesis

A preliminary model comparison between the β_2-adrenergic and h5-HT$_{7(a)}$ receptors was performed and the results showed a strong sequence homology, especially at the level of specific transmembrane helices. Docking of 5-HT into the orthosteric binding cavity of our 5-HT$_7$ model revealed a critical role of residues W7.33, E7.35, Y7.43, R8.53 and D8.54, where some were similar to that previously reported for the 5-HT$_7$ receptor based on ligand docking (Kołaczkowski et al., 2006), and for 5-HT$_{1B}$ and 5-HT$_{2B}$ receptors based on crystallization (Wang et al., 2013). To understand how specific amino acids were involved in the binding process, determination of the 5-HT$_7$ single point mutations was based on the chemical-physical characteristics of the specific amino acid with the objective to change the amino acid charges. In addition, point mutations were made to remove the aromatic groups to determine their importance for the interaction with the specific ligands. By site-directed mutagenesis we generated several clones expressing h5-HT$_{7(a)}$ receptors with single or double mutated amino acids.

Molecular Modeling

Molecular dynamics (MD) simulations and docking studies were carried out using an Intel Core i7 processor, 16 GB RAM, operating under Linux/Ubuntu 10.04. The homology model of the h5-HT$_7$ receptor was kindly supplied by Prof. Ingebrigt Sylte's group (unpublished data) and built from the crystal

structure of the β_2-adrenergic receptor (pdb: 2RH1; Cherezov et al., 2007) by SwissModel server.[1] Conformations of residues that differ between the 5-HT$_7$ and β_2-adrenergic receptors have been optimized using the RefineModel macro of ICM (Abagyan and Totrov, 1994).

The homology model features a disulfide bridge between TMH3 and extracellular loop 2 (ECL2) which are in accordance with the template structure. All the wild type and mutants considered (W7.33V, E7.35T, E7.35R, E7.35D, E7.35A, R7.36V, Y7.43A, R8.52D, D8.53K and R8.52D-D8.53K) were embedded in a bilayer of POPC (palmitoyl-oleyl-phosphatidyl choline; 100 × 100 Å) and solvated with pre-equilibrated water molecules in the three-dimensional space (box of water) of 130 × 130 × 108 Å. The counterions (Na^+ and Cl^-) were placed in the proximity of the regions of the protein surface to mimic an ionic strength of 0.15 mM. Thirty-five nanoseconds of MD simulations were carried out for all the considered systems using the NAMD2 software, version 2.9 (Phillips et al., 2005) with the CHARMM27 force field where all atoms are explicitly represented and water is characterized by the TIP3P model with a dielectric constant of 1 (ε) (Jorgensen et al., 1983). All systems were energy-minimized (conjugate gradient) then gradually heated up to 300 K with a 2-fs time step and equilibrated with a 300 K thermal bath for 400 ps. The velocities were reassigned every 2 ps to achieve complete stability (Berendsen et al., 1984). Production runs were performed at 300 K. The SHAKE algorithm with a tolerance of 1×10^{-8} Å was used to fix the length of the covalent hydrogen bonds (Ryckaert et al., 1977). Non-covalent interactions were calculated at each step. To avoid edge effects and treat long-range electrostatic interactions, periodic boundary conditions and the particle-mesh-Ewald algorithm with a grid size of 130 × 130 × 108 Å (Essmann et al., 1995) respectively, were applied to all of the simulation steps. Non-bonded short-range interactions were treated by a cutoff value of 10 Å. After minimizing the protein, the helices were first equilibrated in water with constraints on the cytosolic side. Next, MD of the helices with added loops and disulphide bonds was performed. Finally, MD of the unconstrained domain embedded in a bilayer POPC was carried out.

The ten mutants of 5-HT$_7$ were constructed based upon the optimized model obtained as described above, adopting the mutate plugin of VMD 1.91. The mutants studied were W7.33V, E7.35A, E7.35D, E7.35R, E7.35T, R7.36V, E7.35T-R7.36V, Y7.43A, Y7.43T, Y7.43F, R8.52D, D8.53K, and R8.52D-D8.53K. 30 ns of MDs calculation for each mutant was performed, using computational resources granted from the supercomputing HPC-CINECA and UK-NSC. The predicted trajectories were analyzed using the VMD 1.91 software (Humphrey et al., 1996). The average structures as extracted from the last xyz atomic coordinates (50 ps for each MD simulation) were used as input file for docking.

Semiflexible docking was carried out by the molecular docking algorithm MolDock Optimizer and the scoring function MolDock [GRID] as implemented in the Molegro Virtual Docker software, version 6 (Thomsen and Christensen, 2006). Only torsion angles in the side chains were modifed during the minimization; all other properties, including bond lengths and backbone atom positions, were held fixed, and a new receptor conformation was generated for each pose after each docking calculation. The 10 runs for each molecule were carried out with a population size of 50, maximum iteration of 2000, scaling factor of 0.50. 5-carboxamidotryptamine (5-CT) and SB269970 structures were built and minimized by the software SYBYL-X 1.3.[2]

Site-Directed Mutagenesis

All mutant h5-HT$_{7(a)}$ sequences used in this work were obtained by site-directed mutagenesis of the coding region of the human h5-HT$_{7(a)}$ sequence cloned in pcDNA3.1 vector (Krobert et al., 2001). We used the QuikChange® Site-Directed Mutagenesis kit containing a *Pfu*Turbo DNA polymerase (Agilent). All mutated sequences were confirmed by DNA sequencing. Using this strategy we generated a set of h5-HT$_{7(a)}$ clones carrying a single mutation at the following positions: W7.33V, E7.35T, E7.35R, E7.35D, E7.35A, R7.36V, Y7.43A, Y7.43F, Y7.43T, R8.52D, D8.53K. We also generated h5-HT$_{7(a)}$ clones with the double mutations E7.35T-R7.36V and R8.52D-D8.53K.

Expression of Wild-Type and Mutant Human 5-HT$_{7(a)}$ Receptors and Cell Culture

The human 5-HT$_{7(a)}$ receptor mutants obtained were expressed in QBI-HEK293 cells which were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO) with 10% fetal bovine serum (BioWhittaker) and penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were transiently transfected with wild-type and mutated plasmid DNA using LIPOFECTAMINE™—LTX reagent (Invitrogen™) and 7.5 μg DNA per 150 mm dish and a serum-free medium (ULTRAculture, BioWhittaker; because serum contains high concentration of serotonin) supplemented with L-glutamine (2 mM), penicillin (10000 U/ml) and streptomycin (10000 U/ml).

Membrane Preparation

Membrane preparations from transiently transfected QBI-HEK293 cells containing the mutated receptors were prepared 48 h after transfection as described previously (Krobert et al., 2001).

Binding Assays

The receptor expression level of QBI-HEK293 cells expressing mutated h5-HT$_{7(a)}$ was determined by radioligand binding. Binding assays were performed in 96-well, round-bottom microtiter plates with total reaction volume of 50 μl, containing the indicated concentration of ligand ([^{3}H]5-CT (serotonin agonist) or [^{3}H]SB269970 (serotonin antagonist)) as previously described (Krobert et al., 2001). Specific binding was defined as the difference between total binding and non-specific

[1]http://swissmodel.expasy.org/

[2]http://www.certara.com

binding (obtained in the presence of 10 μM 5-HT). G-protein-coupled receptors may exist in different conformations, such as low and high affinity states. In the presence of excess GTP most receptors will exist in the low affinity state. To avoid bias due to unpredictable ratios between the two affinity states, GTP (100 μM) was included in the assay mix.

Adenylyl Cyclase Assays

Adenylyl cyclase activity was measured by determining conversion of [α-^{32}P]ATP to [^{32}P]cAMP in membrane preparations as previously described (Krobert et al., 2001). Briefly, adenylyl cyclase activities were measured on 10 μl aliquots in a final volume of 50 μl in the presence of 0.1 mM [α-^{32}P]ATP, 4 mM $MgCl_2$, 20 μM GTP, 1 mM EDTA, 1 mM [^{3}H]cAMP, 1 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma), a nucleoside triphosphate regenerating system and additives (FSK (100 μM), 5-CT, 5-HT and SB269970). The samples were incubated for 20 min at 32°C. Cyclic AMP formed was quantified by the double column chromatography system on Dowex 50 cation exchanger and on neutral aluminium oxide (Alumina) columns. The recoveries of each sample through the columns are monitored by adding [^{3}H]cAMP to the assay mix, thus eliminating individual differences between the columns.

Binding and Adenylyl Cyclase Data Analysis

Binding and adenylyl cyclase data were analyzed by non-linear regression using Microsoft Excel 2007 with the Solver add-in, as described (Krobert et al., 2001).

Western Blot

Lysates of membrane preparations were separated by SDS-PAGE and electroblotted as described (Norum et al., 2005). The membranes were incubated overnight at 4°C with a 1:200 dilution (v/v) of rabbit anti-5-HT$_7$R (Oncogene Research Products, Boston, MA) in PBS containing 5% (w/v) non-fat dry milk and 0.05% (v/v) Tween 20. Thereafter, the blots were incubated with a 1:5000 dilution (v/v) of HRP-linked anti-rabbit IgG (Amersham ECL™-HRP Linked Secondary Antibodies, GE Healthcare). The immobilized HRP-conjugated secondary antibodies were visualized with the LumiGLO Chemiluminescent Substrate (KPL, Inc.) and visualized with a UCP Sensicam (UVP Inc., CA, USA).

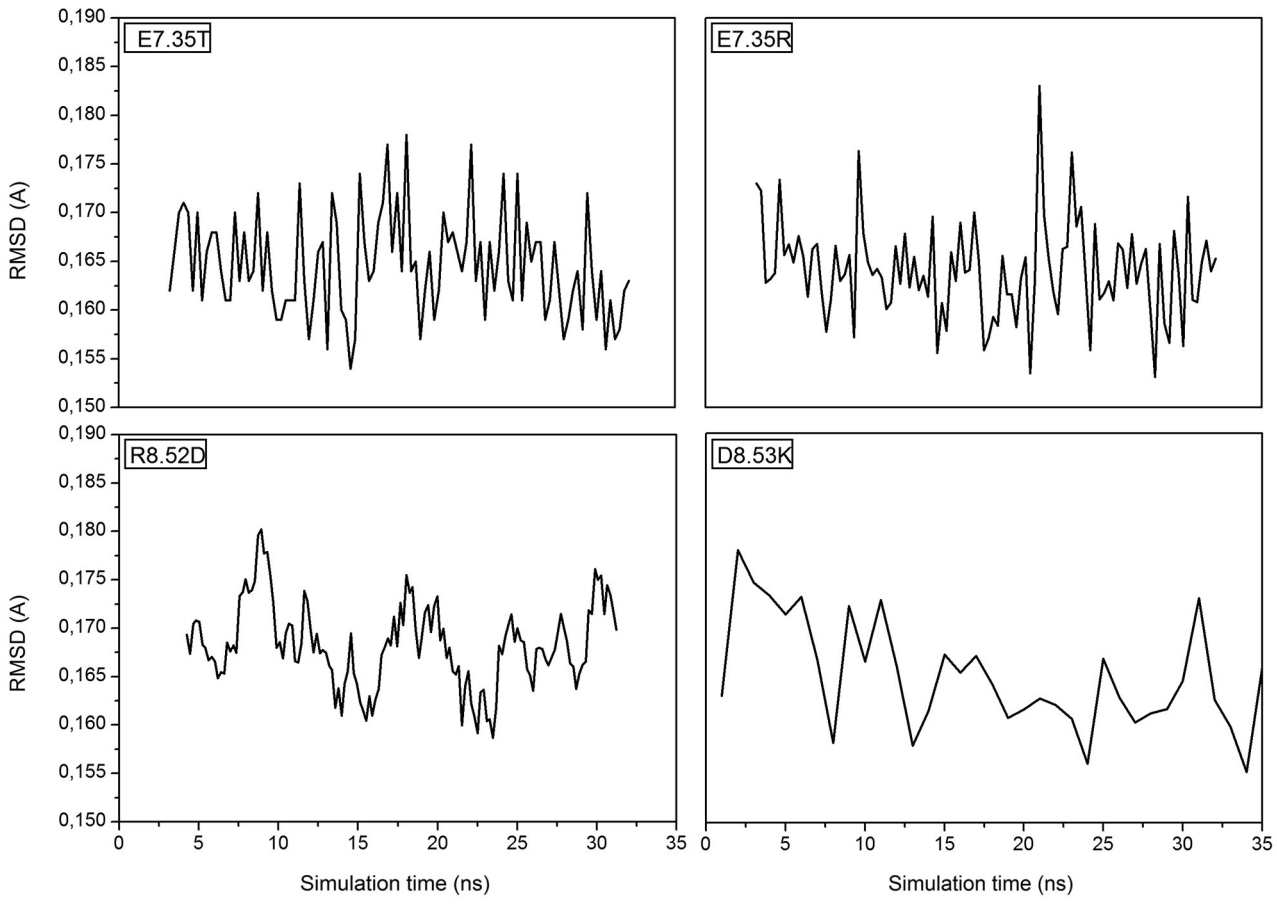

FIGURE 1 | Root Mean Square Deviation (RMSD) is referring to the backbone of the four mutants E7.35T, E7.35R, R8.52D and D8.53K.

Results

Modeling

MD simulations of the mutants of 5-HT$_7$ were performed in order to obtain a reliable structure for the subsequent docking calculation. After about 30 ns of equilibration root mean square deviation (RMSD) profiles were analyzed for all mutants to verify the backbone stability and perform docking analysis. All RMSD curves are steady along the whole equilibration phase and no noteworthy oscillations were observed, demonstrating that all structures were stabilized and equilibrated by 35 ns of MD. **Figure 1** shows the trend of RMSD for backbone atoms of the mutated receptors E7.35T, E7.35R, R8.52D and D8.53K. All analyzed models showed a high structural rigidity; for all models the RMSD was about 0.165 Å.

In addition, in **Figure 2**, the secondary structures of the models were plotted over time, in order to verify that the predicted secondary structure does not change significantly. The backbone of the seven trans-membrane domains seems to be stable in helix conformation during the simulation. On the other hand, residues of the extracellular loops appear to be more flexible, and they do not have a stable conformation. At the end of 30 ns of equilibration, our model consists of seven transmembrane helices (**Figure 2**) and a large cavity defined by the helices III, V, VI and VII, in accordance with Wang et al. (1993). Comparison of our 5-HT$_7$ model with the X-ray structure of 5-HT$_{1B}$ (4IAQ) (Wang et al., 2013) and 5-HT$_{2B}$ (4IB4) (Wacker et al., 2013) receptors, confirms that the main folding pattern of our model appears to be consistent with experimental data, as reported in panel A of **Figure 3**. While the three structures here compared have slightly different primary sequences (e.g., T6.46 in 5-HT$_7$, M6.46 in 5-HT$_{2B}$ and A6.46 in 5-HT$_{1B}$), the same region with different amino acids has the same spatial orientation of the side chains, as evidenced in **Figure 3B**. Particularly, glutamic acid is conserved in all the models, along with its orientation. Moreover, in our model a formation of a hydrogen bond between T7.35 and R6.58 is observed, as discussed later in the discussion section, which should play a role in ligand binding. All the data indicate that our models appear good candidates for further study.

Docking results have been summarized in **Table 1**. All the mutants are predicted to bind both the agonist 5-CT and the antagonist SB269970. **Tables 2**, **3** list the favorable and unfavorable interactions of the two reference molecules with the wild type and mutant receptors. Overall, the antagonist (SB269970) forms a higher number of positive interactions and no unfavorable steric interactions that allow the formation of more stable complex (more negative docking score) than the

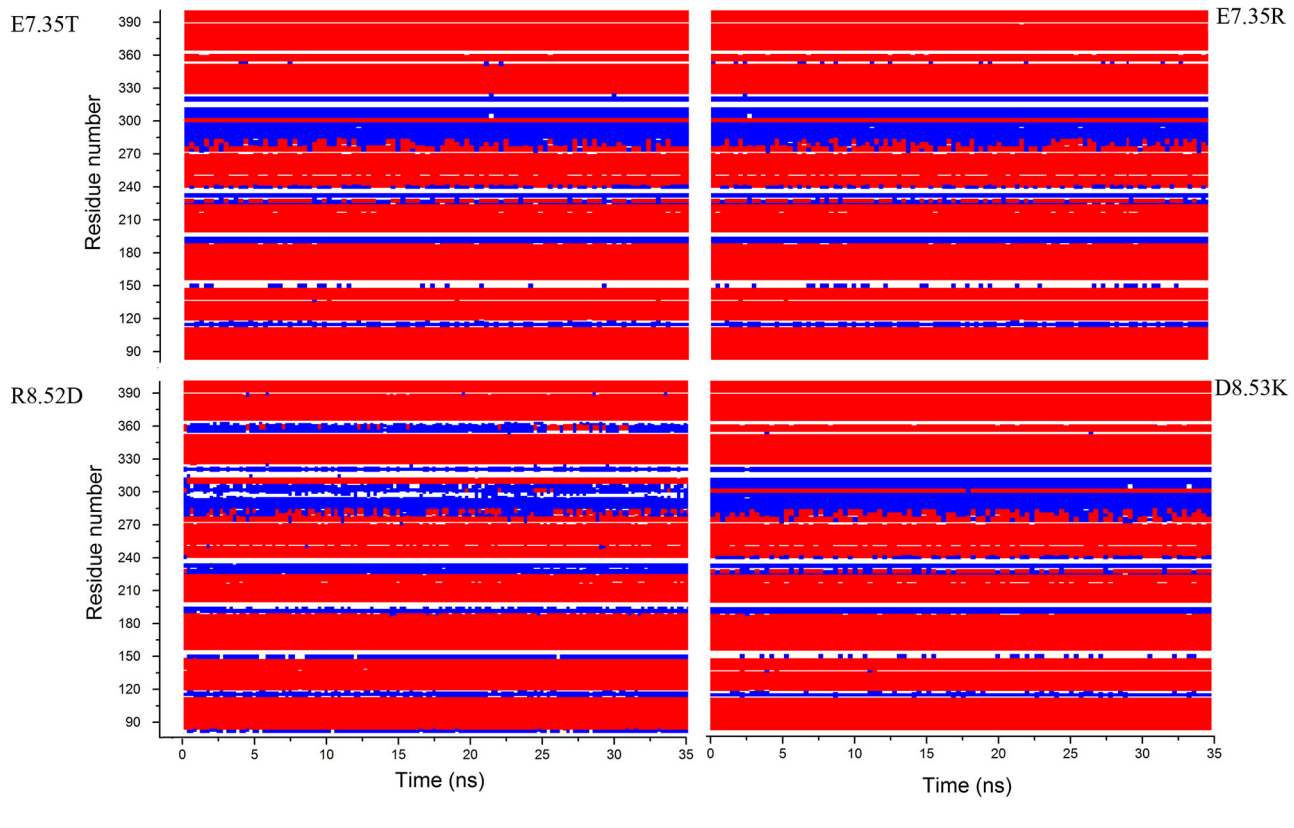

FIGURE 2 | Secondary structure for the mutants E7.35T, E7.35R, R8.52D and D8.53K calculated over the molecular dynamic (MD) trajectory. Red represents helix conformation, blue represents turn conformation, white represents random coil conformation.

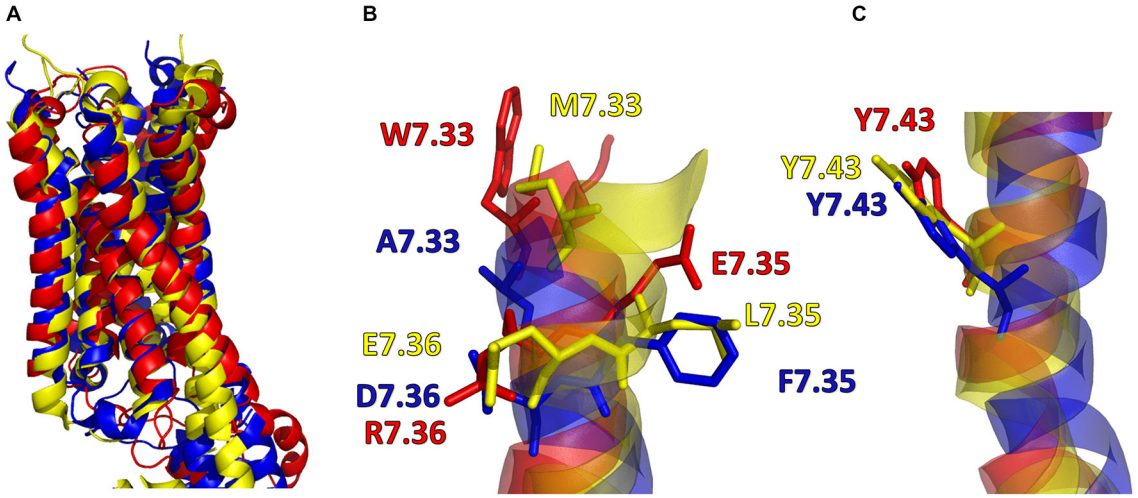

FIGURE 3 | (A) Superimposition of 5-HT$_7$ after 30 ns of equilibration (red) with 5-HT$_{1B}$ (blue) (Wang et al., 2013) and 5-HT$_{2B}$ (yellow) (Wacker et al., 2013). **(B,C)** Main chain of the three receptors represented as stick and aminoacid residues as lines.

agonist. Mutation of E7.35 to Asp, Thr and Arg were predicted to produce better binding compared to the wild type protein, while mutation of E7.35 to Ala was predicted to maintain high binding capacity. On the contrary, mutation of R8.52 to Asp was predicted to produce weaker binding. Only mutant D8.53K was predicted to show slightly lower binding. In this case the increased steric hindrance of the mutations plays a key role in binding properties.

Characterization of Human 5-HT$_{7(a)}$ Mutants

Ligand Binding

The mutant receptors were examined by comparing the binding properties and ability to activate adenylyl cyclase (cyclic AMP production), using transiently transfected QBI-HEK293 cells. The affinities of the agonist (5-CT) and the antagonist (SB269970) determined from saturation binding experiments with up to 3 nM of [^{3}H]5-CT and up to 2.5 nM [^{3}H]SB269970 varied from being not modified, reduced or, in some mutants, no specific binding was detected. The receptor density of all the analyzed mutants was only slightly reduced in a subset of mutants, so receptor density is unlikely to account for changes in affinity (shown in **Table 4**). In addition, the ability to activate AC was either unmodified or reduced in the mutated receptors compared to wild-type (**Table 5**). Most importantly, the mutagenesis studies revealed critical roles of residues E7.35, R7.36 and Y7.43 in ligand binding and R8.52 in activation of AC.

TABLE 1 | MolDock score (a.u.) from docking analysis of wild type and mutant receptors with 5-CT and SB269970.

	5-CT	SB269970
5-HT$_{7(a)}$	−93	−132
W7.33V	−95	−127
E7.35T	−102	−129
E7.35R	−103	−151
E7.35D	−98	−127
E7.35A	−100	−124
R7.36V	−109	−144
Y7.43A	−100	−126
R8.52D	−92	−111
D8.53K	−89	−119
R8.52D-D8.53K	−114	−119

Initially, E7.35 was mutated to Thr to remove the negative charge in this part of the receptor and this new receptor showed a complete lack of ability to bind either agonist or antagonist. To understand which kind of interaction is critical in this part of the receptor for the ligand-receptor-interaction, other mutations were performed at E7.35: (1) to Asp (E7.35D) to understand the importance of amino acid charge; (2) to Ala (E7.35A) to test if the length of the side chain is important for ligand binding; and (3) to Arg (E7.35R) to determine if there are electrostatic interactions involved. These new mutants (E7.35D, E7.35A, E7.35R) were analyzed as above in the binding assays. While the E7.35R mutant receptor was completely unable to bind either agonist or antagonist, the E7.35A mutant displayed reduced binding affinity for agonist, but not for antagonist, and the E7.35D mutation did not change the ability of the receptor to bind any ligands (**Table 4**). Mutation of the neighboring residue, R7.36V, resulted in decreased binding affinity for agonist only and a reduced receptor expression. The double mutant E7.35T-R7.36V was unable to bind any ligands.

The mutation R8.52D, where the positive charge was replaced by a negative one, did not alter the affinity for 5-CT or SB269970 (but the receptor expression was significantly reduced), neither did the mutation D8.53K nor the double mutant R8.52D-D8.53K. Another TMH7 mutation evaluated was Y7.43A, where the aromatic group of residue Y7.43 was replaced with the non-polar Ala residue. This mutation caused a significant

TABLE 2 | List of favorable and unfavorable interactions after docking between SB269970 and the target molecules.

Protein	H bond interactions	Hydrophobic interactions	Favorable steric interaction
Wild type	Cys231, Ile233, E7.35, T2.64, R7.36	I3.29, Leu232, Leu236, L7.39, F2.60, F3.28 (pi stacking), V2.61, Val230	R6.58, S6.55
E7.35A	T4.57, S5.42, Gln235	A5.46, I4.56, Ile233, L4.61, F5.47, F6.52, P4.60, T3.37, Trp221, V3.33	D3.32, C3.36, T5.39, T5.43
E7.35D	D3.32, S6.55	I3.29, Ile233, Leu232, L4.61, F3.28, F6.51, F6.52 (pi-stacking), T5.43 (t-stacking), W6.48, V3.33	R6.58, C3.36, Gln235
E7.35R	W6.48, Y7.43	A5.46, I3.29, Ile233, Leu232, L7.39, F3.28, F6.44, F6.51, F6.52, W6.48, Y7.43, V2.53, V3.33	D3.32, C3.35, C3.36, Cys231
E7.35T	I4.56, T5.43	A5.46, I4.56, Ile233, L4.61, L7.39, F5.47, F6.51, F6.52, T3.37, T4.57, T5.39, T5.43, W6.48, V3.33, V5.45	D3.32, C3.36, Gln235, S5.42
R8.52D	C3.36	A5.46, L6.49, L7.39, M3.34, F5.47, F6.44, F6.51, F6.52 (t-stacking), T3.37, T5.43, W6.48 (t-stacking), V3.33	D3.32
V7.33W	C3.36, D3.32	A5.46, I3.29, I3.40, L7.39, F5.47, F6.51, F6.52, T3.37, T4.57, T5.43, W6.48 (pi-stacking), Y7.43, V2.53, V3.33	S5.42, S6.55
Y7.43A	S6.55	I3.29, Ile233, Leu232, L7.39, F3.28, F6.51, F6.52 (pi-stacking), W6.48 (t-stacking), V3.33	R6.58, D3.32, C3.36, S6.55
R7.36V	G7.42	A2.49, G7.42, L7.39, L7.41, F5.47, F6.51, F7.38, W6.48, W7.40, Y7.43, V2.53	D3.32, C3.35, C3.36, C6.47, S3.39, S7.46
D8.53K	F6.52	A5.46, Ile233, I5.40, L4.61, F4.62, F6.52, P4.60, P6.59, Y5.38, Y5.48, V3.33	Gln223, Gln235, S5.42, S6.55, T4.57, T5.39, T5.43, T6.56
R8.52D-D8.53K	T4.57, T3.37	A5.46, I3.40, I4.56, L7.39, F6.51, F5.47, F6.52, T5.39, W6.48, Y7.43, V3.33	D3.32, C3.36, S5.42, T3.37, T4.57, T5.43

AAs which are part of the helices are numbered according to Ballesteros-Weinstein nomenclature (Ballesteros and Weinstein, 1995).

TABLE 3 | List of favorable and unfavorable interactions after docking between 5-CT and the target molecules.

Protein	H bond interactions	Hydrophobic interactions	Favorable steric interactions
Wildtype[(1)]	I3.29, D3.32, Y7.43, Cys231	A3.30, Ile233, Leu232, L7.39, F3.28 (stacking), V2.61, V2.57, V3.33	R6.58, E7.35
E7.35A	I4.56, V3.33, Gln235, T3.37	A5.46, Ile233, L4.61, P4.60, F6.52, T5.43, Trp221, Y5.38, V3.33, V5.45	S5.42, T4.57
E7.35D	S6.55, Cys231, I3.29	A3.30, Leu232, Ile233, F3.28 (t-stacking), F6.51, V3.33, Val230	R6.58, D3.32, Gln235
E7.35R	D3.32, Y7.43	A3.30, I3.29, Ile233, Leu232, L7.39, F3.28, F6.51, F6.52, W6.48, Y7.43, V3.33	Cys231
E7.35T	A5.46, T5.43, Gln235, D3.32	A5.44, A5.46, I3.40, M3.34, F5.47, F6.52 (t-stacking), V3.33	C3.36, S5.42, T3.37, T5.39
R8.52D	T4.57, I3.29	A3.30, I3.29, Ile233, L4.58, L4.61, P4.60, S5.42, Trp221, Y5.38, V3.33, Val225	Asn224, Gln223, Gln235, T4.57
V7.33W	D3.32, T5.39, T5.43, T3.37	A5.46, I3.40, L4.61, F5.47, F6.52, Y5.38, V3.33	C3.36, Gln235, S5.42, T3.37, T4.57, T5.39, T5.43
Y7.43A	S6.55, T5.43	A5.46, Ile233, F5.47, F6.52, P6.59, Y5.38 (t-stacking), V3.33	Gln235, Ser234, S5.42, S6.55, T4.57, T5.39, T6.56
R7.36V[(2)]	L7.39, W6.48, G7.42, S7.46	G7.42, L6.49, L7.39, L7.41, F5.47, F6.51, F6.52, F7.38, W6.48, W7.40, Y7.43 (t-stacking), V2.53	C3.36, C6.47, D2.50(electro-static interaction), S7.46
D8.53K	Gln235, Ser234	Ala222, Gly220, I3.29, Ile233, L4.61, Leu232, F4.62, P4.60, Trp221, Y5.38, Val225	Asn224, Gln223, Gln235, Ser234, T5.39
R8.52D-D8.53K	A5.46, T4.57, I4.56, S5.42, T3.37	A5.46, I3.40, I4.56, F5.47, F6.52, V3.33	C3.36, S5.42, T3.37, T4.57, T5.39, T5.43

AAs which are part of the helices are numbered according to Ballesteros-Weinstein nomenclature (Ballesteros and Weinstein, 1995). [(1)] *Unfavorable electrostatic interactions with R6.58 and Lys229.* [(2)] *Unfavorable electrostatic interactions with R6.58. Favorable electrostatic interaction with D2.50.*

reduction in agonist binding affinity and a possible reduction in antagonist binding affinity, in addition to a reduction in receptor expression. To determine if the aromatic group or OH-group was involved in the changes elicited by mutation Y7.43A, mutant receptors Y7.43F and Y7.43T were constructed. Preliminary data indicate that Y7.43T showed a reduction in ligand potency and receptor expression, whereas Y7.43F was without effect upon ligand binding.

Effect upon Adenylyl Cyclase Activation

We next examined the effect of the different mutations within TMH7 of the h5-HT$_{7(a)}$ receptor on the transductional response (G protein activation) by measuring their ability to activate adenylyl cyclase (AC). As shown in **Table 5** and **Figure 4**, the W7.33V, E7.35D, R7.36V, Y7.43A, and D8.53K mutated receptors stimulated AC activity with the same potency as the wild-type receptor. A possible higher affinity for the antagonist

TABLE 4 | Binding properties of wild-type and mutant 5-HT$_{7(a)}$ receptors expressed in QBI-HEK293 cells.

Mutant	[^{3}H]5-CT		[^{3}H]SB269970	
	pK$_d$	B$_{max}$ (pmol/mg protein)	pK$_d$	B$_{max}$ (pmol/mg protein)
5-HT$_{7(a)}$	9.51 ± 0.05	2.00 ± 0.27	9.51 ± 0.57	1.79 ± 0.27
W7.33V	9.57 ± 0.11	1.94 ± 0.09	9.30 ± 0.07	1.92 ± 0.21
E7.35T	No specific binding		No specific binding	
E7.35R	No specific binding		No specific binding	
E7.35D	9.17 ± 0.09	2.14 ± 1.01	9.50 ± 0.06	2.08 ± 0.63
E7.35A	8.74 ± 0.32 *	1.14 ± 0.03	9.41 ± 0.01	1.36 ± 0.15
E7.35T-R7.36V	No specific binding		No specific binding	
R7.36V	8.17 ± 0.54 *	0.66 ± 0.37 †	9.77 ± 0.03	0.26 ± 0.06 †
Y7.43A	8.55 ± 0.29 *	0.21 ± 0.09 †	8.98 ± 0.34	0.27 ± 0.16 †
Y7.43T	8.55	0.21	8.56	0.41
Y7.43F	9.21	2.78	9.62	2.52
R8.52D	9.17 ± 0.04	0.53 ± 0.06 †	9.09 ± 0.01	0.46 ± 0.07 †
D8.53K	9.30 ± 0.07	1.22 ± 0.15 †	9.25 ± 0.09	1.09 ± 0.04
R8.52D-D8.53K	9.57 ± 0.09	1.20 ± 0.07	9.53 ± 0.10	1.19 ± 0.07

*Wild-type (5-HT$_{7(a)}$) and mutant receptors were transiently transfected in QBI-HEK293 cells. Membranes were subjected to saturation binding analysis using [^{3}H]5-CT and [^{3}H]-SB269970 as selective ligands. Affinity values are presented as pK$_d$ values (−log Kd). Data shown are average ± SEM of 3–5 experiments, except for Y7.43T and Y7.43F (n = 1). * − P < 0.05 One-way ANOVA with Dunnett's multiple comparisons test vs. wild-type. † − P < 0.05 One-way ANOVA with Dunnett's multiple comparisons test vs. wild-type within experiments.*

TABLE 5 | Activation of adenylyl cyclase (AC) by the agonists 5-CT and 5-HT and inhibition of 10 μM 5-HT-stimulated AC by the antagonist SB269970.

Mutant	pEC$_{50}$		pK$_i$
	5-CT	5-HT	SB269970
5-HT$_{7(a)}$	7.82 ± 0.07	7.06 ± 0.03	8.90 ± 0.10
W7.33V	7.99 ± 0.16	7.27 ± 0.12	8.76 ± 0.31
E7.35T	N.D.	N.D.	N.D.
E7.35R	N.D.	N.D.	N.D.
E7.35D	7.67	6.85	9.12
E7.35A	7.39	6.48	8.77
E7.35T-R7.36V	N.D.	N.D.	N.D.
R7.36V	7.89	6.69	9.67
Y7.43A	7.62 ± 0.28	6.76 ± 0.04 *	8.13 ± 0.03 *
Y7.43T	7.45	5.98	7.28
Y7.43F	8.15	7.23	8.98
R8.52D	N.D.	N.D.	N.D.
D8.53K	7.69 ± 0.06	6.81 ± 0.10	8.59 ± 0.13
R8.52D-D8.53K	6.91 ± 0.07	6.28 ± 0.08	9.15 ± 0.16

*Potency values are presented as pEC$_{50}$ (−log EC$_{50}$) values for agonists and pK$_i$ (−log K$_i$) values for antagonists, calculated by the method of Cheng and Prusoff (1973). Data shown are average ± SEM of 1–8 experiments. N.D., no AC activity detected. *p < 0.05 One-way ANOVA with Dunnett's multiple comparisons test vs. wild-type.*

SB269970 of the R7.36V mutant receptor measured in AC assays (**Figure 4**; **Table 5**) was not supported by binding data (**Table 4**) and therefore not investigated further. Particularly interesting was the finding that the R8.52D mutant which displayed unaltered binding affinity for both agonist and antagonist, was not able to activate AC constitutively (no effect of SB269970), or by either of the agonists 5-CT or 5-HT (**Table 5**). In contrast, the Y7.43A mutant displayed a lower binding affinity for 5-CT, a lower potency on AC for 5-HT and SB269970, but no change in potency to activate AC for 5-CT (**Table 5**). In line with the lack of binding, the mutants E7.35T and E7.35R showed no ability to activate AC, whereas the conservative mutation E7.35D showed no change in AC activation and the E7.35A mutant activated AC with reduced potency.

Lack of Binding not due to Lack of Receptor Expression

To determine if the complete inability of the E7.35T and E7.35R mutants to bind the radioligands [^{3}H]5-CT and [^{3}H]SB269970 resulted from the absence of receptor expression, we determined if these receptors were in fact expressed by subjecting membrane preparations to SDS-PAGE and determining 5-HT$_7$ expression using a polyclonal antibody directed against the N-terminus of the h5-HT$_7$ receptor. In those receptors analyzed (even in mutants with reduced or absent radioligand binding), a protein band of about ∼50 kDa, corresponding with the size of the h5-HT$_7$ receptor, was detected, whereas in non-transfected control cells no band was detected (data not shown). These data indicate that the absence of ligand binding of the various mutant receptors does not result from a lack of receptor expression despite displaying an absence of radioligand binding.

Discussion

GPCRs are challenging targets in drug design (Overington et al., 2006). Despite the recent surge of GPCR structural data, structures have only been determined for a minor fraction of GPCRs. Therefore, *in silico* tools are key to obtain structural information to integrate and rationalize the design of experimental studies of receptor-ligand interactions. Homology modeling has been successfully applied for several different GPCRs (see Sandal et al., 2013 and references therein). Combining *in silico* with experimental tools can lead to accurate structural characterization and accelerate drug design. In this work, the mechanism of molecular recognition between the

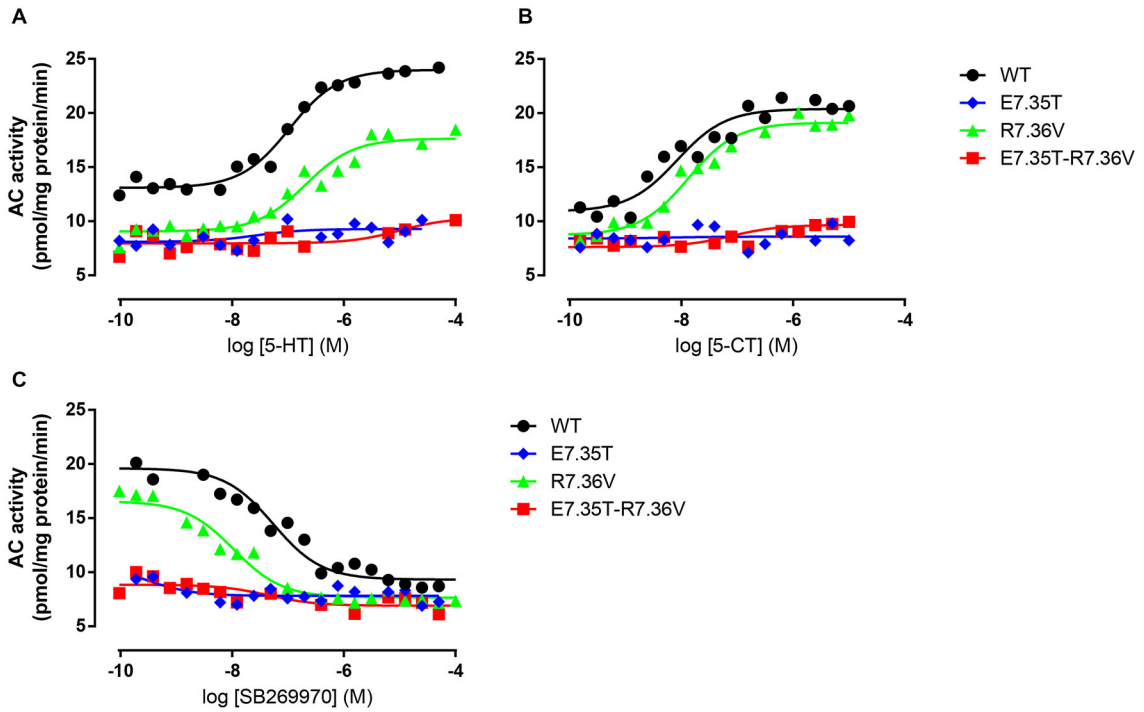

FIGURE 4 | Ability of mutant receptors to activate adenylyl cyclase. Adenylyl cyclase (AC) activity in response to increasing concentrations of 5-HT **(A)** or 5-CT **(B)** in membranes from QBI-HEK293 cells transiently expressing the wild-type (WT) or indicated mutant receptors. In **(C)** the ability of increasing concentrations of SB269970 to antagonize 10 μM 5-HT was determined. AC activity was measured as described under *Materials and Methods*, and the data shown are representative of those obtained from 1–3 independent experiments.

h5-HT$_7$ receptor and ligands was investigated through *in silico* molecular docking and well-established *in vitro* experimental approaches.

The modeling data predicted that the residue E7.35 of the wild type was relevant for ligand binding. In particular, this residue located in the TMH7 is involved in the formation of a three-member salt bridge with R7.36 and R6.58. Therefore, we focused our attention on this residue and performed single point mutagenesis in order to evaluate the effect of specific amino acids on the binding affinity of the receptor. The chosen mutants E7.35T, E7.35R, E7.35A, E7.35D were created by site-directed mutagenesis and analyzed with radioligand binding assays and the ability to activate AC.

The ability to bind both the agonist (5-CT) and antagonist (SB269970) radioligand was completely abolished in the E7.35T mutant, despite expression of the mutant receptor as determined by Western analysis. An analysis of the MD trajectory suggests that the inability to activate the receptor may result from the formation of a H bond between T7.35 and R6.58 in the E7.35T mutant. Possibly, this H bond acts as a locked gate preventing the entry of the ligands to the binding cavity (**Figure 5**). Likewise, no specific binding of either agonist or antagonist ligands was observed in the mutant E7.35R. The MD analysis of E7.35R suggests that the guanidine side chain of R7.35 in the E7.35R mutant moves towards the guanidine side chain of R6.58 blocking the entrance to the orthosteric pocket of the protein (**Figure 6**). Consistent with the lack of binding, the E7.35T and E7.35R mutant receptors were unable to activate adenylyl cyclase activity (**Figure 4**; **Table 5**). Not surprisingly, the conservatively changed mutant E7.35D receptor, maintaining the charged residue, behaved essentially as the WT receptor both regarding binding and AC activation, consistent with the important role of the electrostatic interactions with this residue. Also consistent with this was the finding that the E7.35A mutated receptor, replacing Glu with the nonpolar Ala, showed reduced binding affinity to both agonist (5-CT) and antagonist (SB269970) radioligand (**Table 4**), and was still able to activate AC, but with reduced potency in line with the reduced binding affinity (**Table 5**).

The MD analysis showed that another residue was important in the interaction with the ligands: R7.36. The Arg was changed to Val (R7.36V) and the mutation reduced the affinities of agonist (5-CT), but not for antagonist (SB269970) radioligand (**Table 4**). But the mutant receptor displayed 5-CT-stimulated AC activity with essentially the same properties as the wild-type receptor (**Table 5**; **Figures 4A–C**). MD simulations indicated that the R7.36 is involved in a network of interactions. This residue formed an electrostatic interaction with D2.65, which is partially instable (0.8% of whole simulation), due to the formation, as said before, of a three-member salt bridge R7.36-E7.35-R6.58. Consequently, the extracellular part of TMH7 is constrained by two salt bridges to TMH2 and TMH6.

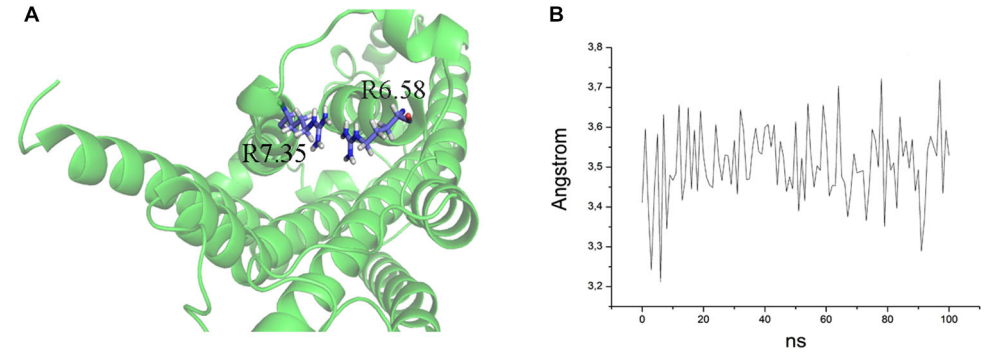

FIGURE 5 | (A) Overlap of R7.35 and R6.58 in the mutant E7.35R. The backbone is shown in cartoon representation and the residues are indicated in stick format. **(B)** Distance between the two guanidine groups of R7.35 and R6.58 measured over the MD trajectory.

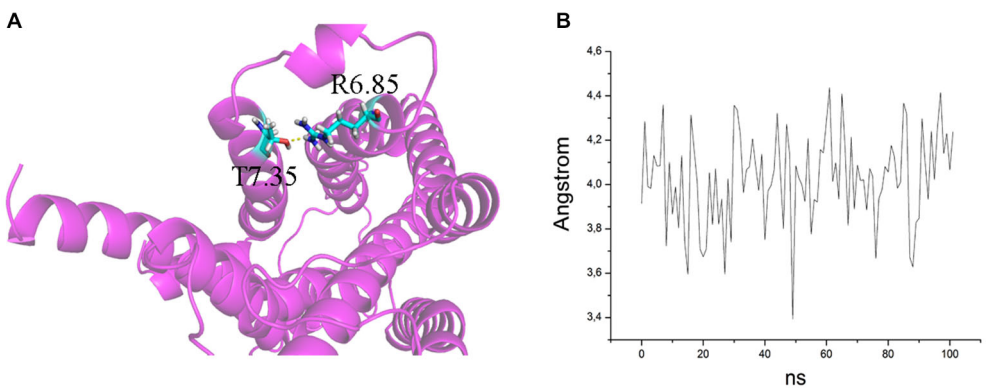

FIGURE 6 | (A) H bond between T7.35 and R6.58 in the mutant E7.35T. The backbone is shown in cartoon representation and the residues are indicated in stick format. **(B)** Distance between the oxygen atom of T7.35 and hydrogen aminic atom of R6.58 measured over the MD trajectory.

The release of this ionic lock could be a key step in receptor activation. In order to test this hypothesis, charge-neutralizing mutations of E5.67 and of D3.49 were made in the β_2-adrenergic receptor (Ballesteros et al., 2001). Experimental data by Ballesteros et al. (2001), together with the high-resolution structure of rhodopsin (Palczewski et al., 2000) suggest that ionic interactions between D/E3.49, R3.50, and E6.30 may constitute a common switch governing the activation of many rhodopsin-like G-protein-coupled receptors. In addition, semi-flexible docking calculations were performed at the binding cavity defined by the residues from the 3rd, 5th, 6th, 7th helices and the ECL2, including an orthosteric pocket embedded in the 7TM core and a long binding pocket close to the extracellular site. The binding mode is in accordance with that recently reported for the crystal structure of the h5-HT$_{1B}$ G-protein-coupled receptor bound to ergotamine or dihydroergotamine, which are accommodated at the orthosteric pocket and an extended binding pocket close to the ECL2, respectively (Wang et al., 2013). The residues belonging to the architecture of this pocket are conserved in 5-HT receptors.

The movements of transmembrane segments (TMs) III and VI at the cytoplasmic side of the membrane play an important role in the activation of G-protein-coupled receptors. There is evidence for the existence of an ionic lock that constrains the relative mobility of the cytoplasmic ends of TM3 and TM6 in the inactive state of the β_2-adrenergic receptor (Ballesteros et al., 2001). The highly conserved R3.50 at the cytoplasmic end of TM3 interacts both with the adjacent D3.49 and with E6.30 at the cytoplasmic end of TM6. Such a network of ionic interactions has now been directly supported by the high-resolution structure of the inactive state of the β_2-adrenergic receptor (Dror et al., 2009) and would serve to constrain the receptor in the inactive state.

The R8.52D mutant receptor showed a different behavior: although the receptor displayed normal affinity for ligands and a significantly reduced expression (**Table 4**), the receptor was unable to activate adenylyl cyclase (**Table 5**). The MD data showed that this residue pointed toward the water during simulation; its position was at the base of helix VII and near the G protein binding domain. We examined the next residue D8.53 to better understand the importance of this region. We changed the Asp to Lys and found that the affinity of both agonist and antagonist ligands for the D8.53K mutant was similar to that observed for the wild-type receptor. A similar result was

obtained in the adenylyl cyclase assays, where the D8.53K mutant receptor activated the enzyme with the same efficiency as the wild-type receptor. However, the D8.53K mutation was able to rescue the ability of the R8.52D-mutated receptor to activate AC, since both 5-CT and 5-HT were able to stimulate adenylyl cyclase activity through the double mutated R8.52D-D8.53K receptor with essentially unchanged potency (**Table 5**) and only a decrease in the efficacy (not shown) compared with the wild-type receptor. Thus the mutagenesis data on the R8.52 residue might be explained by the R8.52D mutation destroying the receptor-G protein interface, possibly by removing the positive charge.

To better understand which kind of interactions are important to bind the specific ligands, to activate the receptor and the downstream pathway, we analyzed the aromatic residues W7.33 and Y7.43. The W7.33 was replaced with Val and Y7.43 with Ala. The mutated receptor W7.33V didn't change the affinities for 5-CT and SB269970 ligands and stimulated cAMP production with the same efficiency as the wild-type receptor.

The Y7.43A mutant showed a significantly reduced affinity of agonist binding, whereas the reduction in antagonist binding did not reach significance. Kołaczkowski et al. (2006) reported this amino acid to be involved in formation of hydrogen bond with ligands. The two mutants Y7.43F and Y7.43T were designed to assess if the aromatic or the OH-group is involved in the binding process. Based on preliminary data, only the mutant Y7.43T showed a reduction in the ability to bind both the ligands. No changes were observed for the mutant Y7.43F (**Table 4**). The data are generally in agreement with that for AC activation and antagonism (**Table 5**). These experimental data were explained by MD simulations, which pointed out that another highly stable salt bridge is formed between Y7.43 and D2.65 in the TMH2 of the 5-HT$_7$ receptor.

The actions of ligands at receptors depend on the affinity for the receptor and the activation of a signaling system, termed efficacy, which is positive for agonists as a result of conformational changes. While some of the above mentioned mutations do not seem to affect ligand recognition, they may still prevent or reduce receptor activation and G protein coupling, illustrating independent contributions of these residues in the WT to stabilizing the bound ligands and/or formation of a ligand-induced active state of the receptor (Osaka et al., 1998; Strange, 2008).

Conclusion

Based on the above reported findings there seems to be a non-ionic lock between helix III and helix VI in the 5-HT$_7$ receptor. However, when the salt-bridge between D3.49 and R3.50 is broken a new one is formed between R3.50 and S2.39. The active and inactive states of the 5-HT$_7$ receptor are characterized by D3.49-R3.50 and R3.50 and S2.39 salt bridges, respectively. The mutant R8.52D in helix VIII lacks cyclase activation. This residue points toward the water during simulation; its position is at the base of helix VII and near the G protein binding domain. Thus, the mutagenesis data on this residue might be explained by destruction of the receptor-G protein interface, and we can assume that the charge is important.

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[3]http://www.glisten-gpcr.eu

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Cellular mechanisms of the 5-HT$_7$ receptor-mediated signaling

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Serotonin (5-hydroxytryptamine or 5-HT) is an important neurotransmitter regulating a wide range of physiological and pathological functions via activation of heterogeneously expressed 5-HT receptors. The 5-HT$_7$ receptor is one of the most recently described members of the 5-HT receptor family. Functionally, 5-HT$_7$ receptor is associated with a number of physiological and pathological responses, including serotonin-induced phase shifting of the circadian rhythm, control of memory as well as locomotor and exploratory activity. A large body of evidence indicates involvement of the 5-HT$_7$ receptor in anxiety and depression, and recent studies suggest that 5-HT$_7$ receptor can be highly relevant for the treatment of major depressive disorders. The 5-HT$_7$ receptor is coupled to the stimulatory G$_s$-protein, and receptor stimulation results in activation of adenylyl cyclase (AC) leading to a rise of cAMP concentration. In addition, this receptor is coupled to the G$_{12}$-protein to activate small GTPases of the Rho family. This review focuses on molecular mechanisms responsible for the 5-HT$_7$ receptor-mediated signaling. We provide detailed overview of signaling cascades controlled and regulated by the 5-HT$_7$ receptor and discuss the functional impact of 5-HT$_7$ receptor for the regulation of different cellular and subcellular processes.

Keywords: serotonergic signaling, G-protein coupled receptors, serotonin 5-HT$_7$ receptor, heterotrimeric G-protein, oligomerization, palmitoylation

GENERAL PRINCIPLES OF G-PROTEIN COUPLED RECEPTOR SIGNALING

G-protein coupled receptors (GPCRs) represent the largest and most diverse superfamily of transmembrane receptors divided into five different families: rhodopsin, secretin, glutamate, adhesion and frizzled receptors (Bjarnadóttir et al., 2006). Initial studies with first discovered GPCRs, bovine rhodopsin and β2 adrenergic receptor, arouse great interest in the field of GPCRs, whose structures and functions became a subject of extensive research (Nathans and Hogness, 1983; Dixon et al., 1986). All these receptors function as signal-transducers by translating extracellular stimuli into intracellular responses resulting in multiple physiological as well as pathophysiological responses (Thompson et al., 2008). All known GPCRs consist of an extracellular amino-terminus, seven membrane-spanning α-helices (for which reason they are often referred to as 7 transmembrane receptors), and an intracellular carboxyl-terminus. Hence GPCR activity is induced by many different ligands, the mechanism of sensing ligands and transducing signals are highly variable (reviewed in Kristiansen, 2004). According to the "allosteric ternary complex model", GPCRs exist in equilibrium between an inactive and active state (Christopoulos and Kenakin, 2002), explaining the agonist-independent, constitutive activity of some receptors (Seifert and Wenzel-Seifert, 2002).

HETEROTRIMERIC G-PROTEINS

Heterotrimeric G-proteins are the main downstream effectors of GPCRs acting as molecular switches by turning on intracellular downstream signaling cascades. They consist of three subunits, α, β and γ and are divided into four subgroups according to the structural and functional similarities of the Gα subunit. The members of the stimulatory Gα$_s$ family stimulate adenylyl cyclases (ACs), whereas inhibitory Gα$_i$ proteins inhibit ACs. The Gα$_q$ class of G-proteins couples to phospholipase Cβ (PLCβ), while Gα$_{12}$ family members activate Rho guanine-nucleotide exchange factors (Rho GEFs; Kristiansen, 2004). To date at least 16 different genes encoding Gα subunits, 5 genes encoding Gβ subunits and 12 different genes encoding Gγ subunits have been discovered. Although not all subunits do interact with each other, the diversity of heterotrimeric G-proteins is still enormous, and this represents an additional level of complexity by the regulation of multiple signaling pathways (Cabrera-Vera et al., 2003).

Heterotrimeric G-proteins become activated by GPCRs *via* complex conformational changes, which are also facilitated by Gβγ dimers (Ford et al., 1998). Upon discovery of the heterotrimeric G-proteins, they were thought to conduct signals exclusively *via* Gα-subunits. Later on, Gβγ dimer has also been shown to directly modulate downstream effectors. First identified downstream target of Gβγ dimer was G-protein coupled inward rectifier potassium (GIRK) channel (Logothetis et al.,

1987). Nowadays, a list of downstream effectors regulated by Gβγ dimers is permanently extending (Woehler and Ponimaskin, 2009).

In parallel with this classical G-protein mediated GPCR signaling, non-classical (G-protein independent) signaling became obvious during the last decade. This type of signaling will be also discussed below.

G-PROTEIN INDEPENDENT SIGNALING

Beside the canonical GPCR signaling pathways *via* heterotrimeric G-proteins, GPCRs can participate in non-canonical, G-protein independent signaling. Main players of the G-protein independent signaling are arrestins - a small family of cytosolic adaptor proteins consisting of four members (Krupnick and Benovic, 1998). In contrast to arrestin 1 and arrestin 4 (X arrestin), which are primary involved in adaption processes of opsins in rods or cones, arrestin 2 and 3 (β-arrestin 1 and 2) are ubiquitously expressed and can interact with different GPCRs (Lefkowitz and Shenoy, 2005). Shortly after receptor stimulation, the C-terminal tail of a GPCR often becomes substrate for the phosphorylation by G-protein coupled receptor kinases (GRKs; Gehret and Hinkle, 2010). Phosphorylated receptors display a high affinity for β-arrestin 1 and 2, which hinder interactions between receptor and heterotrimeric G-protein resulting in desensitization and damping of G-protein dependent signaling (Perry et al., 2002). However, differently than thought at the beginning, arrestins not only switch-off the GPCR-signaling, but can also lead to the activation of alternative signaling pathways. Thus, β-arrestins serve as a signaling hub, linking activated GPCRs to multiple (G-protein independent) signaling pathways such as receptor trafficking as well as in extending GPCR mediated signaling to non-receptor tyrosine kinases (nRTKs) like proto-oncogene c-Src (c-Src) and mitogen-activated protein kinases (MAPK) signaling pathways.

5-HT$_7$ RECEPTOR: PHYSIOLOGICAL FUNCTIONS AND DISTRIBUTION IN THE BRAIN

The 5-HT$_7$ receptor is one of the most recently discovered members of the serotonin receptor family, which was cloned in 1993 independently by researchers in three laboratories (Bard et al., 1993; Lovenberg et al., 1993; Ruat et al., 1993). The 5-HT$_7$ receptor gene is located on human chromosome 10q23.3-q24.3 with an open reading frame containing 1335 base pairs and encoding a protein of 445 amino acids (Bard et al., 1993). The 5-HT$_7$ receptor is broadly expressed in the central nervous system including spinal cord (Dogrul and Seyrek, 2006), thalamus, hypothalamus, hippocampus, prefrontal cortex, and the amygdala where it is expressed in both neurons and glial cells (Hedlund and Sutcliffe, 2004; Thomas and Hagan, 2004; Russo et al., 2005). Significant density of 5-HT$_7$ receptor was observed in raphe nuclei area. In contrast, receptor expression level detected in putamen and cerebellum was relatively low (Horisawa et al., 2013). The 5-HT$_7$ receptor is also expressed in the suprachiasmatic nucleus, and one of the first functions proposed for the 5-HT$_7$ receptor was the regulation of sleep/wake cycles (Lovenberg et al., 1993). Functional analysis demonstrated association of the 5-HT$_7$ receptor with central processes such as learning and memory, including specific aspects of hippocampus-dependent information processing (Hedlund and Sutcliffe, 2004; Ballaz et al., 2007; Eriksson et al., 2008; Gasbarri et al., 2008; Hedlund, 2009). Moreover, 5-HT$_7$ receptor can be implicated in several neurological diseases (Hedlund and Sutcliffe, 2004; Thomas and Hagan, 2004). It has been shown that pharmacological blockade or knock-down of the 5-HT$_7$ receptor induces antidepressant-like behavior in animal models (Guscott et al., 2005; Hedlund et al., 2005; Wesołowska et al., 2007). In addition, certain antidepressants may act directly on the 5-HT$_7$ receptor (Mullins et al., 1999), suggesting this receptor as a novel target by the treatment of depression (Hedlund, 2009; Mnie-Filali et al., 2009). Analysis of mRNA expression level revealed that the amount of 5-HT$_7$ gene transcripts in the dorsolateral prefrontal cortex of schizophrenic patients was increased, demonstrating that 5-HT$_7$ receptor can also be associated with schizophrenia (East et al., 2002; Pouzet et al., 2002; Ikeda et al., 2006).

So far, three splice variants of the 5-HT$_7$ receptor have been identified in human, including 5-HT$_{7(a)}$, 5-HT$_{7(b)}$, 5-HT$_{7(d)}$, three in mouse - 5-HT$_{7(a)}$, 5-HT$_{7(b)}$, 5-HT$_{7(d)}$, and four in rat - 5-HT$_{7(a)}$, 5-HT$_{7(b)}$, 5-HT$_{7(c)}$, 5-HT$_{7(e)}$ (Heidmann et al., 1997; Liu et al., 2001). These splice variants differ only in their short carboxyl-terminal amino acid sequence. Receptor isoforms have altered patterns of tissue distribution, while no difference in their pharmacological properties and coupling to ACs was observed (Heidmann et al., 1997, 1998; Krobert et al., 2001). The human 5-HT$_{7(d)}$ receptor represents an exception, because this isoform possesses a differential pattern of receptor internalization which can affect receptor-mediated signaling (Guthrie et al., 2005). In this regard, 5-HT$_{7(d)}$ receptor was constitutively internalized in the absence of agonist suggesting that its carboxyl-terminal tail, which is the longest among known human 5-HT$_7$ receptor isoforms, may contain a motif that interacts with cellular transport machinery that is distinct from 5-HT$_{7(a)}$ and 5-HT$_{7(b)}$ receptors.

Gα$_s$ SIGNALING MEDIATED BY THE 5-HT$_7$ RECEPTOR

The canonical signaling pathway of the 5-HT$_7$ receptor is activation of G$_s$-protein which in turn can activate different AC isoforms (Shen et al., 1993). ACs show a unique tissue distribution as well as regulatory properties (Krupinski et al., 1989; Bakalyar and Reed, 1990; Premont et al., 1996). *In vitro*, all known AC isoforms are sensitive to the G$_s$ activation (Cooper et al., 1995; Taussig and Gilman, 1995; Sunahara et al., 1996). In contrast, it has been demonstrated that Ca^{2+}/calmodulin-stimulated neural-specific isoforms AC1 and AC8 are insensitive to G$_s$ *in vivo* (Impey et al., 1994; Wayman et al., 1994; Nielsen et al., 1996), and that 5-HT$_{7(a)}$ receptor isoform can stimulate AC1 and AC8 by increasing intracellular Ca^{2+} concentration (Baker et al., 1998). The coupling between 5-HT$_7$ receptor and G$_s$-protein results in increased AC activity leading to production of cAMP, which in turn activates protein kinase A (PKA) thereby inducing phosphorylation of different target proteins (**Figure 1**). This results in activation of multiple downstream signaling cascades, including Ras-dependent and Rap1-independent activation of the neuroprotective extracellular signal-regulated kinases (ERK) and Akt (protein kinase B) pathways (Errico et al.,

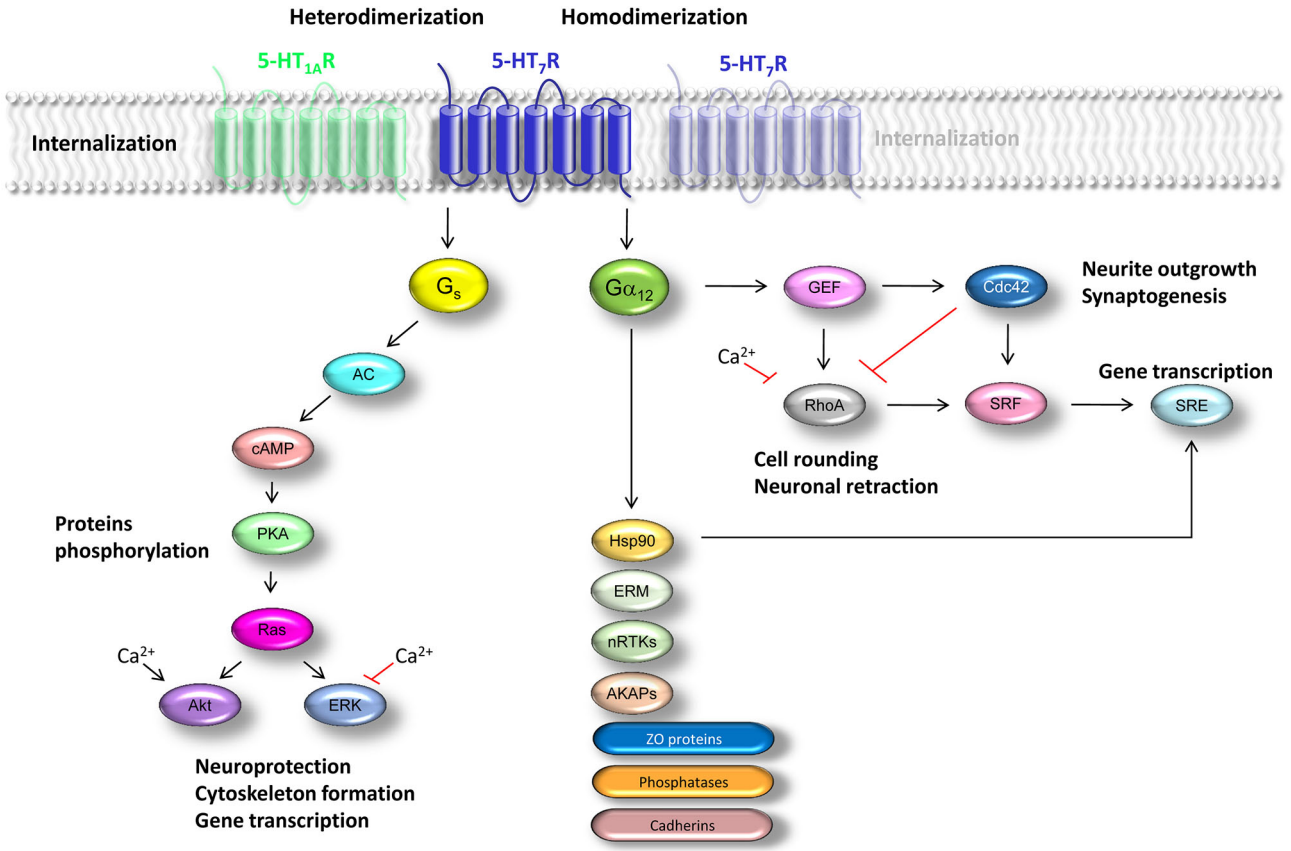

FIGURE 1 | Schematic representation of signaling pathways regulated by the 5-HT$_7$ receptor. Effects mediated by G_s-proteins are in the left section. Summary of the G_{12}-mediated signaling processes is shown in the right section. Abbreviations: GIRK—G-protein coupled inward rectifier potassium channel; AC—adenylyl cyclase; cAMP—cyclic adenosine monophosphate; PKA—protein kinase A; ERK—extracellular signal-regulated kinases; Akt—protein kinase B, Hsp90—heat shot shock protein 90; ERM—proteins of the ezrin-radixin-moesin family; GEF—guanine-nucleotide exchange factor (represented by the leukemia-associated RhoGEF LARG and p115Rho); nRTKs—non-receptor tyrosine kinases; AKAPs—A-kinase anchoring proteins; ZO—zona occludens proteins; SRF—serum response factor; SRE—serum response element.

2001; Johnson-Farley et al., 2005). Noteworthy, 5-HT$_7$ receptor-mediated activation of Akt requires increases both in [cAMP] and intracellular [Ca^{2+}], while activation of ERK is inhibited by Ca^{2+} (**Figure 1**). However, neither an influx of extracellular Ca^{2+} nor release of intracellular Ca^{2+} stores was required for 5-HT$_7$ receptor-mediated activation of ERK in cultured primary hippocampal neurons (Lin et al., 2003). The authors of this study also demonstrated that increase in cAMP concentration causes activation of ERK in neurons *via* a pathway independent of PKA and Raf-1 (Li et al., 1991; Kyriakis et al., 1992). It is widely accepted, that intracellular pathways regulating ERK1/2 and Akt signaling are involved in actin filament reorganization. On the other hand, studies with LM2 cells, which are able to invade into the lung tissue *in vivo*, revealed no significant inhibition in cell motility after Ras-ERK pathway blockade, while PI3K pathways was critically involved in regulation of motility of LM2 cells (Choi and Helfman, 2014). It has been also shown that activation of PI3K activity alone is sufficient to remodel actin filaments and to increase cell migration through the activation of Akt in chicken embryo fibroblast (Qian et al., 2004). Thus, 5-HT$_7$ receptor-mediated activation of G_s-protein can be involved in the activation of effector molecules regulating the cellular motility and cytoskeleton formation.

Gα$_{12}$ SIGNALING MEDIATED BY THE 5-HT$_7$ RECEPTOR

In our previous studies we have demonstrated that 5-HT$_7$ receptor is coupled not only to the G_s-protein, but can also activate G_{12}-protein (**Figure 1**; Kvachnina et al., 2005; Kobe et al., 2012).

The G_{12}-proteins have been shown to activate multiple signaling pathways, and their prominent downstream effectors are members of the Rho family of small GTPases (Rho, Rac, and Cdc42). The G_{12}-protein can modulate the activity of Rho GTPases by activation of guanine-nucleotide exchange factor (GEF) p115Rho which was the first identified downstream effector of Gα$_{12}$ proteins (Hart et al., 1998; Kozasa et al., 1998). Later on, plethora of additional downstream targets of G_{12}-proteins has been discovered. In addition to other RhoGEFs, such as leukemia-associated RhoGEF (LARG) and RhoGEF homologs in *Caenorhabditis elegans*, regulator of G-protein signaling (RGS) family members, proteins

of the ezrin-radixin-moesin (ERM) family, nRTKs, protein phosphatases, A-kinase anchoring proteins (AKAPs), zona occludens proteins and heat shot shock protein 90 (Hsp90) have been identified to directly interact with heterotrimeric G_{12}-protein (**Figure 1**; Hiley et al., 2006; Kelly et al., 2007). The $G\alpha_{12}$ subunit can also interact with C-terminal parts of cadherins leading to release of β-catenin into cytoplasm and nucleus, thus triggering gene transcription (Meigs et al., 2001).

In case of 5-HT_7 receptor, it has been reported that receptor-mediated stimulation of G_{12}-protein results in Rho-dependent activation of a transcription factor, serum response factor (SRF), which binds to the serum response element (SRE; **Figure 1**). Noteworthy, stimulation of 5-HT_7 receptor led to the dose-dependent increase in SRE-driven gene expression even in the presence of a PKA-inhibitor or pertussis toxin (PTX), suggesting a receptor-mediated SRE activation in a PKA-independent manner (Kvachnina et al., 2005). Recent findings also elucidated Rho-independent mechanism of $G\alpha_{12}$-mediated SRE activation via Hsp90 (**Figure 1**; Montgomery et al., 2014). Interaction between $G\alpha_{12}$ and Hsp90 might also be critically involved in a selective transport of the G_{12}-protein to the lipid rafts (Waheed and Jones, 2002).

Detailed analysis of 5-HT_7 receptor-mediated signaling revealed that coupling of receptor to the heterotrimeric G_{12}-protein selectively activates both RhoA and Cdc42 (Kvachnina et al., 2005), suggesting existence of cross-talk between Cdc42 and RhoA pathways. This might be mediated *via* convergent actions of these GTPases on the downstream effector myosin (Manser et al., 1994; Amano et al., 1996). Alternatively, Cdc42 and RhoA may function in a hierarchical cascade wherein Cdc42 downregulates RhoA activity (**Figure 1**; Li et al., 2002).

In neuroblastoma cells, agonist-dependent activation of recombinant 5-HT_7 receptor induces pronounced filopodia formation *via* a Cdc42-mediated pathway paralleled by the RhoA-induced cell rounding (Kvachnina et al., 2005). Stimulation of the 5-HT_7R/G_{12} signaling pathway in cultured hippocampal neurons promotes formation of dendritic spines and accelerates synaptogenesis, leading to enhanced spontaneous synaptic activity (Kobe et al., 2012). Morphogenic action of 5-HT_7 receptor was further confirmed in experiments with striatal and cortical neuronal cultures (Speranza et al., 2013). In this study authors observed pronounced neurite outgrowth after specific activation of 5-HT_7 receptor and demonstrated involvement of ERK and Cdk5 in this process, presuming both proteins to be downstream signaling molecules of $G\alpha_{12}$ (Speranza et al., 2013).

Noteworthy that 5-HT_7/G_{12} signaling in hippocampus undergoes strong developmental regulation. In organotypic hippocampal cultures from juvenile mice, 5-HT_7R/G_{12} signaling potentiates formation of dendritic spines, increases the basal neuronal excitability and modulates synaptic plasticity. In contrast, in older neuronal preparations, stimulation of 5-HT_7 receptor had no effect on neuronal morphology, synaptogenesis and synaptic plasticity (Kobe et al., 2012). Accordingly, the expression level of both 5-HT_7 receptor and G_{12}-protein in the hippocampus is progressively decreased during postnatal development (Kobe et al., 2012). Thus, 5-HT-induced activation of the 5-HT_7R/G_{12} signaling pathways and the consequent reorganization of the dendritic morphology appear to be a part of the molecular cascade required for the growth of new synapses and the formation of initial neuronal networks, which then become the subject of activity-dependent structural and functional plasticity (Citri and Malenka, 2008; Ibata et al., 2008).

HOMO- AND HETERODIMERIZATION OF 5-HT_7 RECEPTORS

G-protein-coupled receptors were initially assumed to exist and function as monomeric units that interact with corresponding G-proteins in 1:1 stoichiometry. Recent studies revealed the capability of GPCRs to form oligomers (Devi, 2001; Bulenger et al., 2005), and it is now widely accepted that homo- and heterodimerization can represent an additional mechanism regulating GPCR-mediated signaling.

Pharmacological analysis in combination with BRET experiments demonstrated that 5-HT_7 receptor can form homooligomers in recombinant system (Teitler et al., 2010; **Figure 1**). Existence of 5-HT_7 receptor homodimers has also been shown in primary cultures of rat cortical astrocytes (Smith et al., 2011). Homooligomerization of 5-HT_7 receptor at the single-cell level has been further confirmed using two different FRET assays (Renner et al., 2012).

By combined application of biochemical and biophysical approaches we have recently demonstrated that 5-HT_7 receptors can form heterodimers with 5-HT_{1A} receptors both *in vitro* and *in vivo* (Renner et al., 2012; **Figure 1**). From the functional point of view, heterodimerization decreases G_i-protein coupling of 5-HT_{1A} receptor and attenuates receptor-mediated activation of G-protein-gated potassium (GIRK) channels, without substantial changes in the coupling of 5-HT_7 receptor to the G_s-protein. Moreover, heterodimerization significantly facilitated internalization of 5-HT_{1A} receptor, while internalization kinetics of 5-HT_7 receptor was decelerated upon heterodimerization (Renner et al., 2012).

PALMITOYLATION OF THE 5-HT_7 RECEPTOR

Many signaling molecules involved in GPCR-mediated signaling are modified by post-translational modifications (Escribá et al., 2007), such as phosphorylation, ubiquitination, glycosylation, palmitoylation and others. The experiments with mutations of two predicted N-glycosylation sites in 5-$HT_{7(a)}$ receptor (N5Q and N66Q) revealed, that 5-$HT_{7(a)}$ receptor glycosylation neither influence the binding of 5-CT agonist to the receptor, nor the potency or efficacy with respect to activation of second messenger cascades, although a decrease in receptor density is apparent for the non-glycosylated receptor (Gellynck et al., 2012). To date, no data about the phosphorylation or ubiquitination of 5-HT_7 receptor are available.

Covalent attachment of long chain saturated fatty acids (i.e., palmitate) to cysteine residue(s) within the protein *via* a labile thioester linkage (S-palmitoylation) represents a widespread post-translational modification of GPCRs since approximately 80% of all known receptors contain the potentially palmitoylable cysteine residue(s) downstream of their seventh transmembrane domain (Escribá et al., 2007). GPCR palmitoylation is involved in the modulation of different receptor functions from coupling to

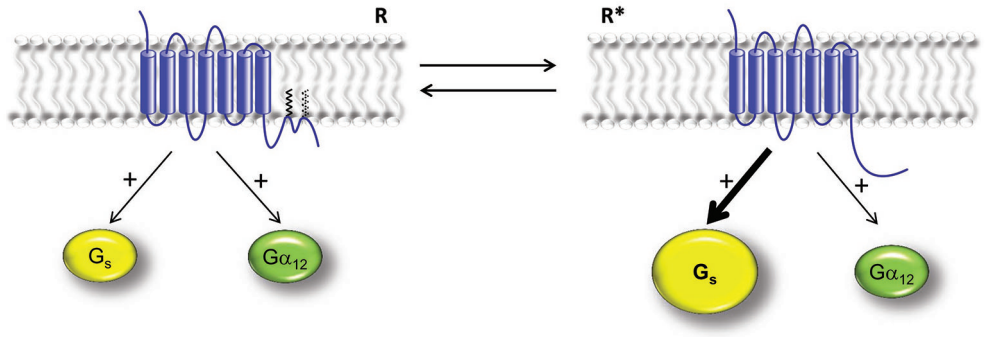

FIGURE 2 | Hypothetical model for the regulation of 5-HT$_7$ receptor activity by dynamic palmitoylation. This model suggests existence of two different receptor populations: Palmitoylated receptors with two additional intracellular C-terminal loops (left) and non-palmitoylated receptors with no intracellular C-terminal loops (right). These populations exist in dynamic equilibrium regulated by basal or agonist-promoted palmitate turnover. Depalmitoylation results in significant increase of the receptor's capacity to convert from the inactive (R) to the active (R*) form in the absence of an agonists. Palmitoylated receptor shows activation of both $G\alpha_s$- and $G\alpha_{12}$-proteins (left). Non-palmitoylated receptor possesses increased agonist-independent, constitutive activity (R*) towards $G\alpha_s$-mediated signaling, while basal receptor-mediated activation of $G\alpha_{12}$-protein is unaltered (right).

G-proteins and regulation of endocytosis to receptor phosphorylation and desensitization. Also the serotonin receptors represent potential substrates for palmitoylation, and palmitoylation was experimentally demonstrated for 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_4$ and 5-HT$_7$ receptors (reviewed in Gorinski and Ponimaskin, 2013).

The mouse 5-HT$_7$ receptor has been shown to undergo dynamic palmitoylation in an agonist-dependent manner after expression in Sf.9 insect cells. Mutation analysis demonstrated that cysteines located in the C-terminal receptor domain at positions 404, 438 and 441 represent the main potential palmitoylation sites (**Figure 2**). Although these cysteine residues were responsible for the attachment of more than 90% of the receptor-bound palmitate, palmitoylation of 5-HT$_7$ receptor was still not restricted to its C-terminus, pointing to the existence of additional acylation site(s) within the receptor.

Functional analysis of palmitoylation-deficient mutants revealed that agonist-induced activation of G_s- and G_{12}-proteins was unaffected. However, mutation of the Cys404 either alone or in combination with Cys438/Cys441 significantly increased the agonist-independent, G_s-mediated constitutive 5-HT$_7$ receptor activity, while the activation of G_{12}-protein was not affected (**Figure 2**; Kvachnina et al., 2009). Generally, these data suggest that palmitoylation of 5-HT$_7$ receptor might be directly involved in the isomerization of the receptor from the inactive to the active form in the absence of agonists. This transformation can be realized by dictating the conformation of receptor's flexible cytoplasmic loops which might be involved either in the receptor/G_s-protein recognition or in G_s-protein binding and/or receptor-mediated G_s-protein activation (**Figure 2**). In combination with the previous findings on the functional role of 5-HT$_4$ receptor palmitoylation (Ponimaskin et al., 2002, 2005), this observation suggests that palmitoylation can represent a general feature regulating constitutive receptor activity. Moreover, in case of 5-HT$_7$ receptor (which is coupled to both, G_s- and G_{12}-proteins) dynamic palmitoylation can represent a molecular mechanism responsible for selective G_s- or G_{12}-mediated signaling.

PHARMACOLOGICAL PROPERTIES OF 5-HT$_7$ RECEPTOR

During the last decade, several selective agonists and antagonists for 5-HT$_7$ receptors have been developed and applied to investigate its pharmacology. Pharmacological analysis revealed that application of risperidone, 9-OH-risperidone, methiothepin, bromocryptine, lisuride, and metergoline resulted in irreversible inhibition of the recombinant 5-HT$_7$ receptor expressed in HEK-293 cells (Smith et al., 2006; Knight et al., 2009). In contrast, action of other potent 5-HT$_7$ receptor antagonists, including clozapine, mesulergine, penfluridol, amperozide and cinanserin is reversible and can be washed out (Knight et al., 2009). In other study receptor-inactivating properties of risperidone, 9-OH-risperidone, bromocriptine, methiothepin, metergoline, and lisuride have been demonstrated. Noteworthy that methiothepin and bromocriptine maximally inhibited forskolin-stimulated adenylate cyclase, whereas the other drugs produced partial inhibition, indicating the drugs are inducing slightly different inactive conformations of the 5-HT$_7$ receptor (Toohey et al., 2009). Nowadays, the highly specific 5-HT$_7$ receptor antagonist SB-269970 (pK_i = 8.9 nM) is a mostly used receptor antagonist for *in vitro* and *in vivo* studies (Kobe et al., 2012; Renner et al., 2012; Tokarski et al., 2012; Vasefi et al., 2013; Guseva et al., 2014; Monti and Jantos, 2014). For the pharmacological activation of the receptor, a high-affinity receptor agonist 5-CT (IC_{50} = 0.83 nM, EC_{50} 13 nM) is widely used in a numerous *in vitro* and *in vivo* studies (Guscott et al., 2003; Kobe et al., 2012; Vasefi et al., 2013). However, 5-CT is known to activate 5-HT$_{1A}$, 5-HT$_{1B}$, and 5-HT$_{1D}$ receptors. Therefore, analysis of 5-HT$_7$ receptor functions by 5-CT requires parallel application of 5-HT$_{1A/1B/1D}$ receptor antagonists. Recently, various novel selective agonists such as AS-19, LP-44, LP-12, LP-211 and E-55888 were developed in addition to 5-CT (reviewed in Di Pilato et al., 2014). Amongst them two novel agonists, LP-211 and LP-378, have been

investigated in regard to exploratory motivation, anxiety-related profiles, and spontaneous circadian rhythm (Adriani et al., 2012). The authors have shown that three- to four-fold dosage of LP-378 was necessary to induce the same effect as LP-211. The latest studies, both *in vitro* and *in vivo*, indicated LP-211 (K_i = 379 nM) as a more specific 5-HT_7 receptor agonist with great potential for future investigations (Speranza et al., 2013; Monti and Jantos, 2014).

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Activation of 5-HT7 receptor stimulates neurite elongation through mTOR, Cdc42 and actin filaments dynamics

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Recent studies have indicated that the serotonin receptor subtype 7 (5-HT7R) plays a crucial role in shaping neuronal morphology during embryonic and early postnatal life. Here we show that pharmacological stimulation of 5-HT7R using a highly selective agonist, LP-211, enhances neurite outgrowth in neuronal primary cultures from the cortex, hippocampus and striatal complex of embryonic mouse brain, through multiple signal transduction pathways. All these signaling systems, involving mTOR, the Rho GTPase Cdc42, Cdk5, and ERK, are known to converge on the reorganization of cytoskeletal proteins that subserve neurite outgrowth. Indeed, our data indicate that neurite elongation stimulated by 5-HT7R is modulated by drugs affecting actin polymerization. In addition, we show, by 2D Western blot analyses, that treatment of neuronal cultures with LP-211 alters the expression profile of cofilin, an actin binding protein involved in microfilaments dynamics. Furthermore, by using microfluidic chambers that physically separate axons from the soma and dendrites, we demonstrate that agonist-dependent activation of 5-HT7R stimulates axonal elongation. Our results identify for the first time several signal transduction pathways, activated by stimulation of 5-HT7R, that converge to promote cytoskeleton reorganization and consequent modulation of axonal elongation. Therefore, the activation of 5-HT7R might represent one of the key elements regulating CNS connectivity and plasticity during development.

Keywords: 5-HT7 receptor, actin dynamics, axonal elongation, Cdc42, mTOR, neurite outgrowth

Abbreviations: 5-HT, 5-hydroxytryptamine; 5-HT7R, serotonin receptor 7; Cdc42, cell division cycle 42; Cdk5, cyclin-dependent kinase 5; CTX, cortex; E, embryonic age; ERK, extracellular signal-regulated kinases 1/2; HIPP, hippocampus; mTOR, mammalian target of rapamycin; p70S6K, p70 S6 kinase; STR, striatal complex.

Introduction

The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) modulates a variety of physiological processes in the Central Nervous System (CNS). In addition to its well-established role in neurotransmission, 5-HT has been shown to regulate the connectivity of the brain by modulating cellular migration and cytoarchitecture during development (Daubert and Condron, 2010). Mammalian brain contains extensive serotonergic projections that exert their effects through at least 14 different subtypes of 5-HT receptors, classified into 7 different families (Pytliak et al., 2011). The 5-HT receptor 7 (5-HT7R) is the most recently identified 5-HT receptor. This seven-transmembrane G-protein-coupled receptor is positively linked to adenylate cyclase through the stimulatory Gs protein and Gα-12 subunit of heterotrimeric G-protein (Kvachnina et al., 2005; Leopoldo et al., 2011), and it is expressed in CNS and periphery. In human and rodent CNS, high densities of 5-HT7R have been found in hypothalamus, thalamus, hippocampus, cortex, amygdala and striatal complex. This distribution is highly correlated with 5-HT7R functions in the CNS, namely circadian rhythm, REM sleep, thermoregulation, learning and memory and nociception (Matthys et al., 2011; Adriani et al., 2012). Consistent with such a broad range of functions, its deregulation has been associated with numerous pathological processes of the CNS, such as obsessive—compulsive disorder (OCD), anxiety, schizophrenia, epilepsy, migraine, sensation-seeking behavior, impulsivity and depression (Cates et al., 2013; Gellynck et al., 2013).

Although precise biological roles 5-HT7R are not well known yet, recent studies suggest that this receptor might be implicated in the modulation of synaptic wiring in the CNS. Interestingly, 5-HT7R expression was markedly increased in adolescent rodents administered with methylphenidate. These animals display specific changes in brain reward circuits and in the reward-based behavior, suggesting that 5-HT7R may play a major role in the modulation of self-control behavior by supporting the persistent brain structural rearrangements during postnatal development (Adriani et al., 2006; Leo et al., 2009). Indeed, it has been shown that 5-HT7R modulates hippocampal neuronal morphology, excitability and plasticity, by participating in dendritic morphogenesis and synaptogenesis (Kobe et al., 2012; Tajiri et al., 2012). In line with these results, pharmacological stimulation of 5-HT7R with a new selective agonist, LP-211 (Hedlund et al., 2010), has been successfully employed to rescue the synaptic plasticity deficits in hippocampal slices from a mouse model of the X-fragile syndrome (Costa et al., 2012) and to alleviate the behavioral phenotype in a mouse model of the Rett syndrome (De Filippis et al., 2014). Thus, all these recent findings indicate that 5-HT7R controls neuritogenesis and synaptogenesis, but the signaling mechanisms remain poorly understood.

Recently we have shown that 5-HT7R activation in neuronal primary cultures from rat brain stimulates neurite outgrowth through Cdk5 and extracellular signal-regulated kinases 1/2 (ERK) pathways, with the modifications of selected cytoskeletal proteins (Speranza et al., 2013), supporting the hypothesis that the 5-HT7R might play a crucial role in shaping neuronal networks during development (Volpicelli et al., 2014). The modulation of connectivity in the nervous system requires remodeling of neuronal morphology and synaptic connections, which in turn depend on dynamic reorganization of cytoskeleton. Various extracellular cues induce changes of the actin cytoskeleton to remodel the structure and the function of subcellular regions by way of numerous signaling pathways, and dynamic changes of actin filaments are crucial for neurite outgrowth and axonal pathfinding and synaptogenesis (Schaefer et al., 2008). One of the most characterized actin-binding proteins, cofilin, binds, twists and severs actin filaments enabling remodeling of the actin cytoskeleton (Bernstein and Bamburg, 2010; Bravo-Cordero et al., 2013). Cofilin is regulated by the upstream signaling pathways involving Rho/Rac/Cdc42 GTPase; in turn, the Rho signaling pathway and the consequent cofilin-dependent actin polymerization are, in many cells, under the control of the mammalian target of rapamycin (mTOR; Jaffe and Hall, 2005; He et al., 2013). The latter is a multifunctional serine/threonine protein kinase which also plays important roles in neurite elongation, dendritic spines shape and synaptogenesis (Guo et al., 2011; Briz and Baudry, 2014; Takei and Nawa, 2014).

In this work we have first demonstrated that the stimulation of 5-HT7R with the selective agonist LP-211 (Hedlund et al., 2010) leads to enhanced neurite outgrowth in embryonic neuronal primary cultures derived from the cortex (CTX), hippocampus (HIPP) and striatal complex (STR) of embryonic mouse brain corroborating the results that we have previously obtained in rat brain (Speranza et al., 2013). As an extension of this finding, we have herein analyzed several intracellular transduction pathways connecting the stimulation of 5-HT7R to neurite elongation. We have found that, in addition to ERK phosphorylation and Cdk5 activation, mTOR and Cdc42 are activated during the 5-HT7R-dependent neurite elongation. With all these pathways converging in the modulation of the dynamics of neuronal cytoskeleton proteins, our data indicate that signals activating 5-HT7R is transduced to neurite elongation via remodeling of actin cytoskeleton. In addition, by use of the microfluidic chambers, we have demonstrated for the first time that the neurites outgrowing in response to 5-HT7R stimulation include axons, suggesting an unexpected role of this receptor in axonal pathfinding and regeneration.

Materials and Methods

Neuronal Primary Cultures

Timed pregnant C57 BL/6 mice were housed, cared for and sacrificed in accordance with the recommendations of the European Commission. All the procedures related to animal treatments were approved by Ethic-Scientific Committee for Animal Experiments and Italian Health Ministry. The embryonic age (E) was determined by the date of insemination (i.e., the appearance of vaginal plug was considered as day E0). For every cell culture preparation, about 15–20 embryos from different dams were pooled. The STR and CTX from E15 embryos, and the HIPP from E18 embryos were dissected under a stereoscope in sterile conditions, and placed in PBS without calcium and magnesium, supplemented with 33 mM glucose. Cells dissociated from embryonic STR, CTX, and HIPP were cultured as previously

described (di Porzio et al., 1980). Briefly, the dissected areas were enzymatically dissociated by incubation for 30 min at 37°C in a papain solution (Warthington, 20 U/ml, Milan, Italy) in Earle's balance salts containing 1 mM EDTA (Sigma-Aldrich, Milan, Italy), 1 mM cysteine (Sigma-Aldrich) and 0.01% pancreatic DNAse (Sigma-Aldrich). 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 cell count, cell suspension was diluted 1:1 with 0.1% trypan blue dye (Sigma-Aldrich) 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 $1.5 \times 10^5/cm^2$ in 2 cm^2 Lab-Tek chamber slides (Nunc) for morphological analyses, and at a density of $3 \times 10^5/cm^2$ 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 dissolved in water (Sigma-Aldrich).

Cultures were grown in serum-free Neurobasal medium (Life technologies, Milan, Italy), supplemented with B27 (Life technologies), 2 mM L-glutamine (Sigma-Aldrich), penicillin (50 U/ml, Sigma-Aldrich) and streptomycin (50 μg/ml, Sigma-Aldrich). Cells were maintained for 3 days *in vitro* (DIV) at 37°C in a humidified incubator in presence of 5% CO_2, before experimental manipulation. For each experimental point, cultures were prepared at least in independent triplicates, and were repeated using distinct culturing sessions.

Drugs and Reagents

The cell cultures were treated with 100 nM of the selective 5-HT7R agonist LP-211 (gift of M. Leopoldo, University of Bari, Italy), 100 nM of the HT7R antagonist SB-269970 (Tocris, Milan, Italy; Hagan et al., 2000), or with a combination of these drugs. Roscovitine (Sigma-Aldrich), a Cdk5 inhibitor, was used at the final concentration of 20 μM. The mTOR inhibitors rapamycin (Sigma-Aldrich) and torin 1 (Tocris), were used at a final concentration of 20 and 250 nM, respectively. ZCL 278 (Tocris), a selective inhibitor of Cdc42, was used at a final concentration of 50 μM. Cytochalasin D (Sigma -Aldrich) was used at a final concentration of 100 nM, while latrunculin and jasplakinolide (Molecular Probes, Milan, Italy) were used at a final concentration of 2 μM. Cells were pretreated for 30 min with 10 μM of U0126, the ERK 1/2 inhibitor, as recommended by manufacturer (Cell Signaling, Milan, Italy). Drugs were added to cultures 72 h after cell plating and incubated for appropriate time.

RNA Isolation and RT-PCR Analyses

Total RNA was extracted from primary cells cultured in 4 cm^2 wells, 3 days after seeding, using the Tri-Reagent according to the manufacturer's instructions (Sigma-Aldrich). The analyses were always carried out in triplicate samples for each experimental point. Reverse transcriptase and quantitative real time PCR analyses were performed as described in Speranza et al. (2013). Primer sets used were:

5-HT7R: Fw GCGGTCATGCCTTTCGTTAGT—Rev GGCGATGAAGACGTTGCAG;

HPRT: Fw TGGGAGGCCATCACATTGT—Rev AATCCAGCAGGTCAGCAAAGA.

Gene expression levels were quantified by the comparative threshold cycle (*CT*) method (Schmittgen and Livak, 2008) using hypoxanthine phosphoribosyltransferase (HPRT) as an internal control gene. The fractional number of PCR cycles (*CT*) required to obtain a given amount of amplified product in the exponential phase of amplification was determined for the gene of interest and for HPRT in each cDNA sample. The relative expression level of the gene of interest was then expressed as $2^{-\Delta CT}$ where $\Delta CT = CT$ gene of interest—*CT* HPRT.

Morphological Characterization and Analysis of Morphometric Parameters

For morphological characterization of neuronal cultures, cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), for 30 min at room temperature (RT), washed three times in PBS, and then permeabilized for 20 min in PBS containing 0.1% Triton-X-100 and 10% normal goat serum (NGS). Cells were treated with blocking solution [10% NGS, 0, 1% bovine serum albumine (BSA) in PBS] at RT for 1 h and incubated with the primary antibody in antibody solution (0, 1% BSA in PBS) overnight at 4°C. The following antibodies were used at the indicated dilutions: monoclonal antibody against neuron specific class III β-tubulin (Tuj1, Covance, Milan, Italy) 1:500 and polyclonal antibody 5-HT7 receptor (Imgenex, Milan, Italy) 1:70. The cells were washed in PBS, and then incubated with fluorescent secondary antibodies (Alexa Fluor goat anti-rabbit, and Alexa Fluor Goat goat anti-mouse, Life technologies) diluted 1:400 in antibody solution.

Cells were then counterstained with DAPI (nuclear stain, 1:1000) for 10 min, washed with PBS and mounted with oil mounting solution (Mowiol). Fluorescent signals from Tuj1 stained neurons were detected with a microscope (Leica DM6000B) equipped with an objective 20x. Images were acquired with high-resolution camera using the software Leica Application Suite, and were analyzed by the image-processing software Image J, for the perimeter and the area of the soma, neurites number and length. In each image, the neuronal cells were recognized by their immunoreactivity with Tuj1 antibody. Using the Image J software the images were pre-processed to optimize illumination and contrast. The length of the neurites was estimated by measuring the length of a line manually drawn from the soma to the end of the primary neurites (neurites that originate directly from soma) using the "Measure" function of the software (modified from Hannan et al., 2014; **Figure 2A**).

Only clearly visible cells were subjected to analysis to prevent inaccurate scoring. A total of 15 fields for each cell-culture condition was selected from at least three independently treated culture wells. A total of 300 neurites/well was traced from Tuj1 positive neurons to measure their length. The analyses were carried out blindly to avoid any subjective influences during the measurements.

Morphometric parameters were always compared to the controls from the same batch of dissociated cells treated with vehicle

alone for the same time length (CTRL). Significant agonist-induced increase in neurite length varied always between 1.2 and 2 fold compared to CTRL. For easy comparison of the results among various cell preparations, data were expressed as percentage of the average CTRL.

Incubation and Immunofluorescence in Microfluidic Chambers

Hippocampal neuron cultures were prepared from 18-day old (E18) mouse embryos. The hyppocampi were dissected in cold HBSS 1X and 3 mM HEPES (Life Technologies), containing antibiotics (100 unit/ml penicillin and 0.1 mg/ml streptomycin, Sigma-Aldrich), and transferred into a 15 ml tube. After a rinse in buffer, specimens were incubated, for 30 min at 37°C, with 40 μl/HIPP of 0.5% trypsin (EuroClone SpA, Pero, Italy) (equivalent to 0.2 mg trypsin/HIPP) and 100 μg/ml DNAase. After an extensive wash, hippocampi were mechanically dissociated and cell density was determined using a counting chamber. Cells were re-suspended in 100 μl of the appropriate medium and plated in microfluidic chambers (Xona Microfluidics LLC, Temecula, California USA; Taylor et al., 2005; Park et al., 2006). Chambers were prepared as suggested by manufacturer's instructions: briefly, 35 mm Petri dishes were coated overnight with 100 μg/ml poly-L-lysine (Sigma-Aldrich) at 37°C and then washed and air dried under a sterile hood. Poly-dimethylsiloxane chambers were placed on the Petri dishes with their micro-channel side down, sealed to the dish by gentle pressure and filled with 20 μg/ml laminin (Sigma-Aldrich), for 2 h at 37°C. Laminin was removed just before plating cells, approximately 100,000 cells were pipetted directly into one compartment (the soma compartment) and then both compartments were filled with Neurobasal medium containing 1X B27 supplement (Life Technologies), 0.5 mM glutamine, 25 μM glutamate and antibiotics. When neurons were plated at this density, very few cells approached the microchannels, so that most likely one axon/channel enters. Cells were exposed to DMSO (control) or DMSO + LP-211, added to both compartments (somatic and axonal), and maintained *in vitro* for 6 days (6 DIV). Medium was changed every 24 h and the number of axons reaching the axonal compartment was blindly counted every day by three operators, from 1 to 6 DIV, at the inverted microscope, in phase contrast, at a magnification of 40x. Finally, at 6 DIV, after the last count, cells were fixed in 4% formaline and 4% sucrose in PBS for 30 min at RT, added directly to both compartments, without removing the microfluidic chambers. After fixation, cells were blocked in 1% BSA (Sigma-Aldrich), 10% normal goat serum (NGS) (Jackson ImmunoResearch Europe Ltd, Suffolk, England) and 0.5% Triton X-100 in PBS, for 1 h at RT, and then incubated overnight at 4°C with the primary antibodies diluted in 1% bovine serum albumin, 1% NGS and 0.2% Triton X-100 in PBS. Antibodies used were: mouse anti-neuron specific βIII-tubulin (Tuj1; Covance, Emeryville, CA, USA, #MMS-435P; 1:3000), in co-localization with either rabbit anti Map2 (AbCam, Cambridge, UK, #32454; 1:400), to visualize dendrites, or rabbit anti Tau (AbCam, #ab64193; 1:50), to visualize axons. Cells were then incubated for 1 h at RT with the appropriate secondary antibodies: goat anti-mouse IgG Alexa Fluor 488 (Molecular Probes, Life Technologies) and goat anti-rabbit IgG Cy3 (Jackson ImmunoResearch), both diluted 1:1000. ProLong Gold Antifade Reagent (Invitrogen, Life Technologies) was added to both compartments to visualize and store immunolabed cells.

Gel Electrophoresis and Western Blot Analyses

For mono-dimensional polyacrylamide gel electrophoresis (PAGE), culture dishes were lysed in RIPA Buffer in presence of protease inhibitors (Roche, Milan, Italy). Proteins (15–30 μg/lane) were separated on 10–12% SDS-polyacrilamide gel and transferred to PVDF membranes (GE Healthcare, Milan, Italy). For experiments with S6 kinase, Akt and cofilin antibodies, culture dishes were lysed in 20 mM MOPS pH 7, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 60 mM β-glycerol-phosphate, 1 μM sodium orthovanadate, 1% triton), in presence of protease inhibitors. Proteins (20–30 μg/lane) were separated as previously described and transferred to PVDF membranes. Filters were blocked for 30 min in 5% (w/v) non-fat milk in Tris-buffered saline Tween-20 (TBST; 0.1% Tween, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) and probed for 2 h at RT with the following antibodies: anti-5-HT7 receptor (Imgenex IMG-368, 1:300), anti-β-actin (BD Transduction Laboratories, #612656, 1:1000), anti-p-ERK1/2 (Cell Signaling, #9101, 1:750), anti-ERK 1/2 (Santa Cruz Biotechnology # sc-93, Milan, Italy1:1000), p70 S6 kinase (Cell Signaling, #2708, 1:1000), phospho-p70 S6 kinase (Thr389; Cell Signaling, #9234, 1:1000), Akt (Cell Signaling, #9272, 1:1000), phospho-Akt (Ser473; Cell Signaling, #4060, 1:1000), cofilin (Cell Signaling, #5175, 1:1000) and p-cofilin (phospho Ser3; Abcam, ab12866, 1:1000). After washing, immunoblots were incubated with goat anti-rabbit IgG (GE Healthcare ECL, 1:10000) or anti-mouse IgG antibodies (GE Healthcare ECL, 1:10000) conjugated to horseradish peroxidase (HRP) and visualized on autoradiographic film, using enzyme-linked chemiluminescence (ECL; Immobilion Western, Millipore). The relative protein levels were determined by densitometry and compared with the protein level of the appropriate standard (β-actin for 5-HT7R blots, p70 S6 kinase for phospho-p70 S6 kinase blots, cofilin for p-cofilin blots, and ERK1/2 for p-ERK1/2 blots) probed on the same membrane, after stripping of the antibody previously used. Net intensity value of each band was calculated by subtracting the background of each area from the total intensity.

For 2D-PAGE, monolayer cultures of striatal and cortical cells, treated or untreated with LP-211, were harvested, washed three times with PBS and lysed in the lysis buffer (40 mM Tris-HCl pH 8.0, 8 M urea, 4% CHAPS, 65 mM DTT, and 1 mM PMSF). Proteins were extracted by repeated freezing and thawing with liquid nitrogen and pooled from 3 independent culture dishes. Samples were centrifuged at 17500 g for 15 min at 4°C to eliminate cellular debris and sonicated into an ultrasonic bath for 15 min. Samples were then centrifuged at 17500 × g for 15 min at 4°C. The supernatant was collected and protein concentration determined by the Bradford method, according to manufacturer's instructions (Bio-Rad, Milan, Italy). Lysates were aliquoted and stored at −80°C until use. 50 μg of lysate proteins were analyzed by 2D-PAGE, as previously described (Speranza et al., 2013). Briefly,

samples to be processed by isoelectrofocusing (IEF) were diluted with the rehydration buffer (8 M urea, 0.5% CHAPS, 0.2% DTT, 0.5% IPG ampholytes, and 0.002% bromophenol blue) to a final volume of 125 μl. The precast IPG strips (3–10 linear pH gradient, 7 cm long, GE Healthcare), used for the first dimension, were passively rehydrated and loaded with the sample at RT for 12 h under low-viscosity paraffin oil. IEF was then performed using an IPGphor isoelectric focusing cell (GE Healthcare), according to the following protocol: 50 V for 3 h, 100 V for 2 h, 500 V for 2 h, 1000 V for 2 h, 3000 V for 2 h, 4000 V for 2 h, 5000 V for 2 h, 6000 V for 2 h, 8000 V until about 25000 V h total. Strips were then equilibrated twice for 15 min under gentle shaking in the equilibration solution (6 M urea, 50 mM Tris-HCl buffer pH 8.8, 30% glycerol, 2% SDS, 0.002% bromophenol blue) containing 1% DTT (to reduce disulfide bonds), in the first equilibration step, and 2.5% iodoacetamide (to alkylate thiols), in the second step. The second-dimension separation was performed on 12% polyacrylamide gels (0.75 mm × 7.5 × 10 cm) by using a Miniprotean II apparatus. The strips were fixed with 0.5% agarose and 0.002% bromophenol blue dissolved in SDS/Tris running buffer. The run was carried out at constant power (10 mA/gel for 15 min; 20 mA/gel until the end of the run). After the second dimension, 2D-gels were electroblotted onto nitrocellulose membranes by using a TransBlot Turbo system (Bio-Rad) following the manufacturer's instructions. The membrane was blocked with 5% milk in TBS-0.1% Tween (TTBS) for 1 h at RT and washed with TTBS. Subsequently, membranes were incubated with primary antibodies diluted in 2.5% milk in TTBS overnight at 4°C. The following antibodies were used: anti-β-actin (BD Transduction Laboratories, #612656, 1:5000); anti-cofilin (Cell Signaling, #5175, 1:1000); β-tubulin (Covance, MMS-435P, 1:1000). Membranes were then washed with TTBS and incubated with the appropriate HRP-conjugated secondary antibody diluted 1:2000 in 2%milk in TTBS for 1 h at RT. Membranes were then washed three times with TBS. Immunoreactive protein bands were visualized by the ECL Plus Western Blotting Detection System (GE Healthcare) according to the manufacturer's instructions.

Statistical Analyses

All the statistical analyses were performed using GraphPad Prism 3.0 (GraphPad Software). Significance of differences was assessed by One Way ANOVA followed by Dunnett *post-hoc* test when drugs-treated cultures were compared to the corresponding control cultures treated with vehicle, or by Tuckey *post-hoc* test for intergroups comparisons. Significance threshold was set at $p < 0.05$.

Results

Neuronal Cultures From Cortex and Striatum of Mouse Brain

As expected from previous data on rat primary cultures (Speranza et al., 2013), the phenotype of cells obtained from the CTX and STR of mouse brains at E15 showed that almost 95% of STR and CTX cells in culture were neurons, as judged by co-localization of Tuj1 antibodies with the nuclear DAPI staining. In addition, about 90% of striatal and cortical neurons were positive to 5-HT7R (**Figure 1A**).

To evaluate the expression levels of the 5-HT7R in our cultures, we first performed real time quantitative RT-PCR analyses. Although the 5-HT7R mRNAs were slightly higher in the STR than in the CTX, the difference was not statistically significant (**Figure 1B**). Accordingly, Western blot analyses showed that the abundance of the 5-HT7R protein normalized to that of β-actin, was similar in both areas (**Figure 1C**).

After 3 days in culture, cells were stimulated with the selective 5-HT7R agonist (LP-211; Hedlund et al., 2010) and stained with the Tuj1 antibodies for morphometrical analysis at various time points, in order to measure the neurite elongation. **Figure 2A** shows representative images of CTRL and LP-211 treated neurons. In line with our previous data (Speranza et al., 2013), we observed that the exposure of neuronal cultures to 100 nM LP-211 caused a significant increase in the length of neurites after 2 h of stimulation in comparison to the control cultures treated with vehicle for the same time length (CTRL, **Figure 2B**). This effect was specifically due to 5-HT7R, as co-treatment of the cells with LP-211 and the 5-HT7R selective antagonist SB-269970 completely abolished the neurite elongation, while addition of the antagonist alone for 2 h had no effect (data not shown).

Signal Transduction Pathways Involved in 5-HT7R-Dependent Neurite Elongation

mTOR Signaling Pathway

Since in neuronal cells mTOR signaling plays a pivotal role in dendritic and axonal growth, we chose to investigate its involvement in neurite elongation stimulated by 5-HT7R. To this aim, we used rapamycin, a drug that interacts with and inhibits the mTOR kinase activity, and torin 1, a specific ATP-competitive mTOR inhibitor. As shown in **Figure 2B**, in cortical cultures the LP-211-induced enhancement of neurite elongation was completely abrogated by the co-treatment with 20 nM rapamycin or 250 nM torin 1. Treatment with rapamycin or torin 1 alone did not affect neurite length. Comparable results were obtained using striatal neurons (data not shown). To further confirm the involvement of the mTOR signaling following agonist stimulation of the 5-HT7R, we investigated the phosphorylation level of the p70 S6 kinase (p70S6K), a direct mTOR downstream substrate (Burnett et al., 1998). To assess the proportion of the phosphorylated form of p70S6K, we performed Western blot analyses using two different antibodies specific for total p70S6K protein (including both phosphorylated and unphosphorylated form) and for the phosphorylated form of the enzyme. The calculated ratio of phospho-p70S6K/ p70S6K was thus used as an index of the mTOR kinase activity. As shown in **Figure 3A**, we found that treatment of neuronal cultures with LP-211 strongly enhanced p70S6K phosphorylation. Treatment with torin 1, alone or in combination with LP-211, completely blocked this phosphorylation, confirming that it is due to mTOR kinase activity.

mTOR interacts with several proteins to form two distinct complexes named mTOR complex1 (mTORC1) and mTOR complex2 (mTORC2), with different upstream and downstream effectors. mTORC2, but not mTORC1, directly activates the protein kinase Akt by phosphorylating its hydrophobic motif (Ser473;

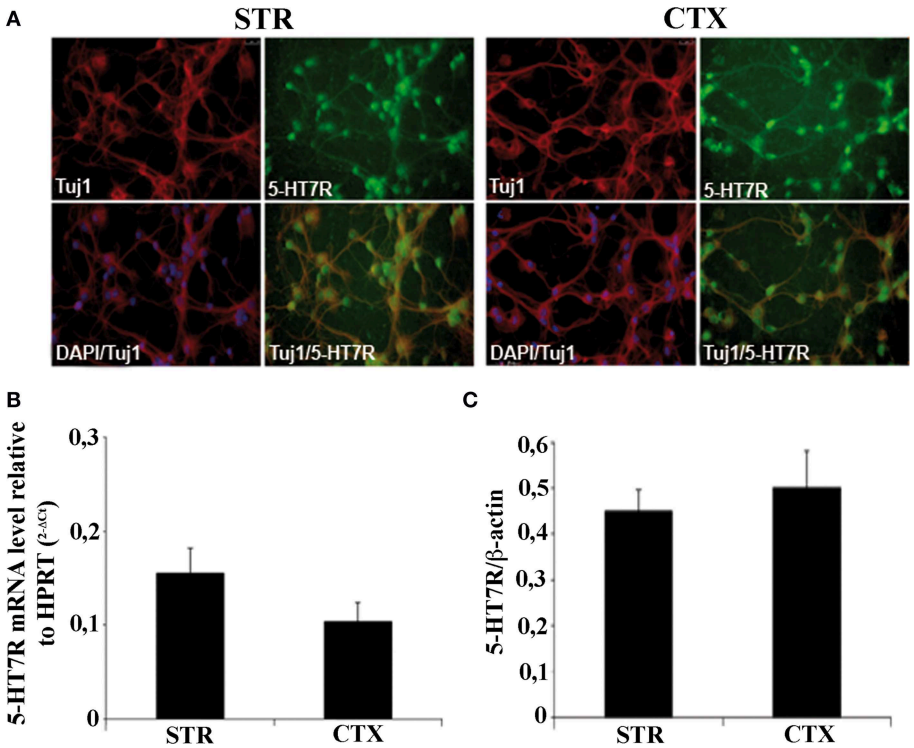

FIGURE 1 | Characterization of striatal and cortical primary cultures. (A) Photomicrographs of the cells in striatal and cortical cultures immunostained with specific antibodies against neuronal marker Tuj1 (red), and 5-HT7R (green), as indicated in each panel. Cell bodies were counterstained with the nuclear marker DAPI (blue). **(B)** Expression levels of 5-HT7R mRNAs, determined by real time RT-PCR, in neuronal cultures. The bars represent the 5-HT7R mRNA levels normalized with those of the housekeeping gene HPRT (means ± SEM; $n = 3$). **(C)** Quantitation of 5-HT7R protein in neuronal cultures. The bars represent the densitometric values of 5-HT7R Western blot signals normalized with those of β-actin signals in the same samples (mean ± SEM; $n = 3$). STR: cultures from the striatal complex of E15 mouse embryos; CTX: cultures from the cortex of E15 mouse embryos.

Sarbassov et al., 2005). Therefore, to discriminate between mTORC1 and mTORC2, we analyzed the phosphorylation levels of Akt at Ser473 by Western blot analyses, using two antibodies specific for total Akt protein (including both phosphorylated and unphosphorylated form) and for the phosphorylated form of the enzyme. The phosphorylated Akt isoform, normalized to the total Akt protein did not change significantly in LP-211 stimulated neurons compared to CTRL, suggesting lack of mTORC2 involvement (**Figure 3B**).

Altogether these results indicate that 5-HT7R-stimulation by LP-211 activates mainly mTORC1 signaling.

Cdc42 Signaling Pathway

Cdc42 is a protein belonging to the Rho/Rac/Cdc42 GTPase subfamily, that plays a key role in regulating axonal and dendrite morphogenesis (Luo, 2000; Auer et al., 2011). To study its possible implication in neurite outgrowth induced by 5-HT7R stimulation, we treated mouse striatal and cortical cultures with ZCL 278, a selective inhibitor of Cdc42. As shown in **Figure 4**, the LP-211-induced enhancement of neurite elongation in cultures from both brain regions was completely abolished by the co-treatment with 50 μM ZLC. It is noteworthy that the treatment with ZCL alone significantly reduced the neurite length in comparison to CTRL of about 10% in striatal cultures and about 20% in cortical cultures. Taken together these data indicate that the Cdc42 signal transduction pathway is involved not only in the enhanced neurite outgrowth induced by LP-211 stimulation of 5-HT7R, but also in time-dependent neurite elongation occurring in basal condition. Thus, this pathway may represent the general signaling system leading to neurite elongation.

ERK and Cdk5 Signaling Pathways

ERK signaling pathway is implicated in neurite outgrowth (Colucci-D'Amato et al., 2003; Jessberger et al., 2009). In addition, we previously demonstrated that in striatal and cortical rat neuronal cultures, the enhanced neurite elongation induced by 5-HT7R stimulation requires ERK phosphorylation and Cdk5 activation (Speranza et al., 2013). Thus, we analyzed the effect of LP-211 on ERK activation also in the striatal mouse neuronal cultures. In line with data on neurite elongation, when neuronal cultures were treated with LP-211 for 2 h, the extent of ERK phosphorylation was significantly increased when compared to CTRL, and this effect was blocked by the co-treatment with 5-HT7R antagonist (SB-269970; Supplementary Figure 1A). These results show that the stimulation of 5-HT7R is responsible for the ERK

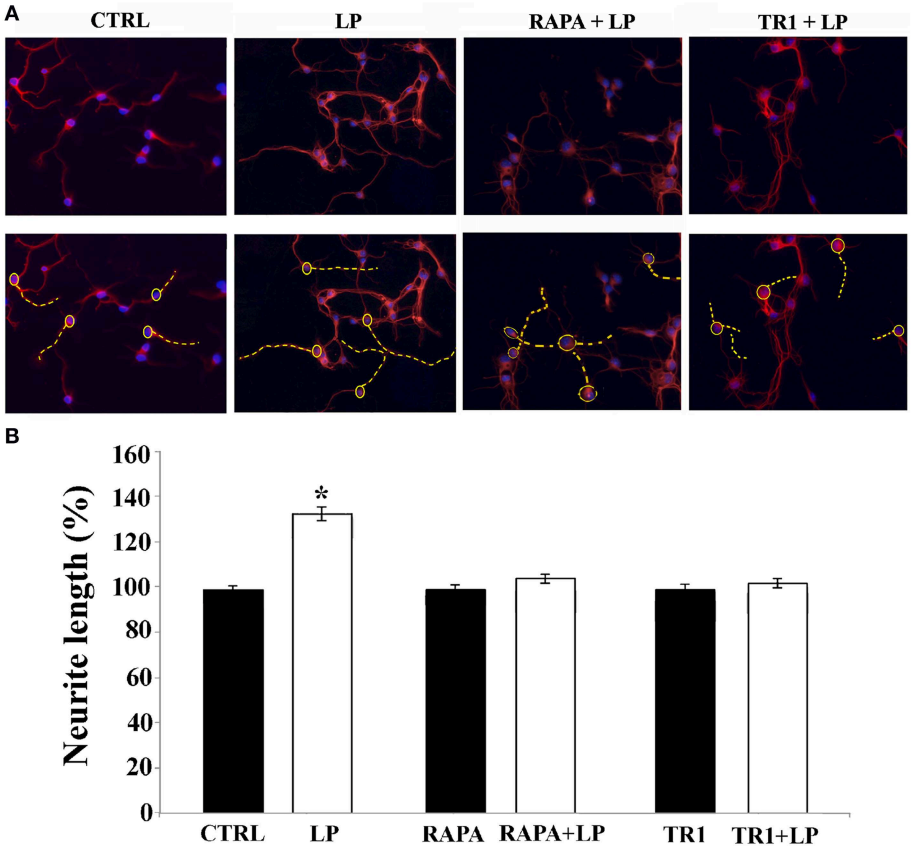

FIGURE 2 | mTOR signaling pathway is required for neurite elongation induced by 5-HT7R stimulation. Cortical neurons were treated for 2 h with the 5-HT7R selective agonist LP-211 (LP, 100 nM), or the mTOR inhibitors rapamycin (RAPA, 20 nM), or torin 1 (TR1, 250 nM) with or without LP. **(A)** Representative Tuj1 immunostaining of neuronal cultures (magnification 20x). The images in the lower row are the same of the upper row with the addition of the dashed yellow lines manually drawn by the operator from the soma (yellow circle) to the end of the primary neurite in order to measure neurite length. **(B)** The graph shows the neurite length expressed as percentage of values measured in the corresponding vehicle-treated cultures (CTRL, set to 100%). The bars represent means ± SEM from randomly selected fields for each cell culture condition ($n = 9$). Asterisk (*): value significantly different from CTRL by One Way ANOVA followed by Dunnett *post-hoc* test ($p < 0.05$).

activation. As expected, the ERK phosphorylation induced by LP-211 was abolished by the treatment with ERK inhibitor, U0126, for 2 h, and U0126 alone also significantly reduced ERK phosphorylation in unstimulated cells (Supplementary Figure 1A). Interestingly, inhibition of ERK phosphorylation by U0126 completely abrogated the enhancement of neurite outgrowth induced by LP-211, while treatment with U0126 alone did not affect neurite elongation (Supplementary Figure 1B).

In line with our previous data (Speranza et al., 2013), we also confirmed that treatment of mouse striatal cultures with roscovitine (inhibitor of Cyclin-dependent protein kinase 5, Cdk5) completely abrogated neurite elongation induced by treatment with LP-211 for 2 h (data not shown).

Activation of 5-HR7 Induces Qualitative and Quantitative Changes of the Actin Cytoskeleton

To establish the relationship between 5-HT7R activation and actin cytoskeleton dynamics, we treated mouse striatal cultures with 100 nM cytochalasin B or 2 μM latrunculin A. These agents, promoting net depolymerization of the actin filaments, nearly abolished the neurite outgrowth stimulated by LP-211, indicating that this enhanced elongation required the polymerization of actin pool (**Figure 5**). When added to the culture media without LP-211, these two compounds significantly reduced the neurites length in comparison with the vehicle-treated cells (CTRL). Accordingly, when the striatal neurons were exposed to the agent promoting actin polymerization (jasplakinolide, 2 μM) the length of their neurites significantly increased (**Figure 5**). Interestingly, when neurons were co-treated with jasplakinolide and LP-211, the effect was not cumulative and the neurites length was similar to the one obtained with LP-211 alone. Collectively, these results indicate that the neurite outgrowth induced by LP-211 depends on modulation of actin cytoskeleton dynamics.

To further investigate the role of the cytoskeleton in LP-211-induced neurite elongation, we have studied the effects of LP-211 on mouse striatal and cortical cultures using 2D protein analyses of β-tubulin, β-actin and cofilin. Immunostaining of 2D-gels of both control striatal and cortical cultures showed a marked stretching of both β-tubulin and actin spots, attesting a high level

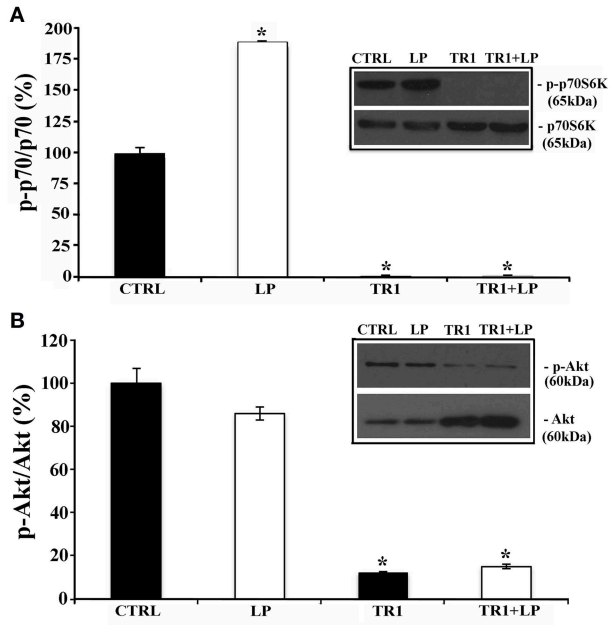

FIGURE 3 | Stimulation of 5-HT7R activates mTORC1 signaling. Cortical neurons were treated for 2 h with the 5-HT7R selective agonist LP-211 (LP, 100 nM), or the mTOR inhibitor torin 1 (TR1, 250 nM), alone or in combination. **(A)** The bars show the level of p70S6K phosphorylation at Thr389 (means ± SEM, $n = 6$), expressed as percentage of values measured in the corresponding vehicle-treated cultures (CTRL, set to 100%). The level of p70S6K phosphorylation was measured by Western blot as intensity of p70S6K phosphorylated at Thr389 (p-p70S6K) normalized with that of total (phosphorylated and unphosphorylated) p70S6K in the same samples. The inset displays representative blots probed with antibodies against p-p70S6K and p70S6K; the molecular weights (kDa) are shown on the right. **(B)** The bars show the level of Akt phosphorylation at Ser473 (means± SEM, $n = 3$), expressed as percentage of values measured in the corresponding vehicle-treated cultures (CTRL, set to 100%). The level of Akt phosphorylation was measured by Western blot as intensity of Akt phosphorylated at Ser473 (p-Akt) normalized with that of total Akt (Akt) in the same samples. The inset displays representative blots probed with antibodies against p-Akt and Akt; the molecular weights (kDa) are shown on the right. Asterisk (*): value significantly different from CTRL by One Way ANOVA followed by Dunnett *post-hoc* test ($p < 0.05$).

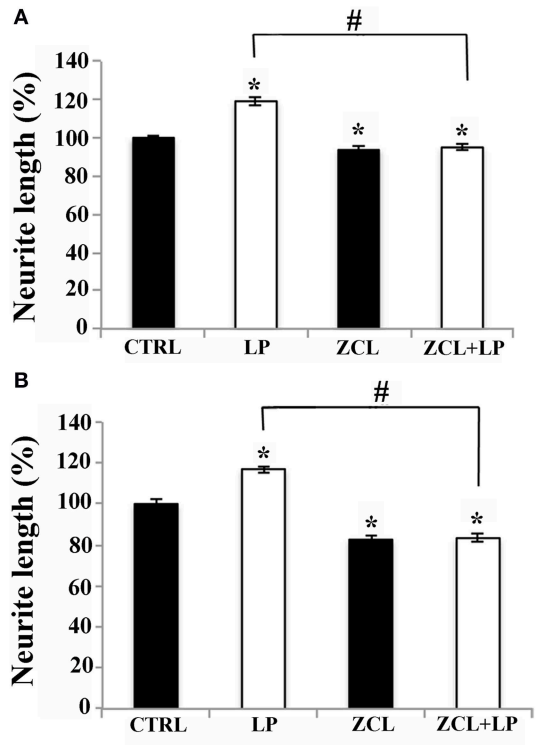

FIGURE 4 | Cdc42 signaling pathway is required for neurite elongation induced by 5-HT7R stimulation. (A) Striatal and **(B)** cortical neurons were treated for 2 h either with the 5-HT7R selective agonist LP-211 (LP, 100 nM), or with the selective Cdc42 inhibitor ZCL (50 μM), or with a combination of the two. The graphs show the neurite length expressed as percentage of values measured in the corresponding vehicle-treated cultures (CTRL, set to 100%). The bars represent means ± SEM from randomly selected fields for each cell culture condition ($n = 6$). Asterisk (*): values significantly different from CTRL by One Way ANOVA followed by Dunnett *post-hoc* test ($p < 0.05$). Number sign (#): values significantly different from each other by One Way ANOVA followed by Tuckey *post-hoc* test ($p < 0.05$).

of protein heterogeneity in terms of post-translational modification. These protein stretches were not substantially affected upon treatment with LP-211 with the exception of a reduction of the actin acidic spots, more marked in striatal neurons (**Figures 6A,C**). Interestingly, LP-211 treatment was found to alter the 2D expression profile of cofilin, a regulator of actin filament assembly/disassembly, in striatal and cortical cells. Indeed, a shift of the stretch of cofilin spots at 21 kDa toward acidic pH region was observed following LP-211 treatment.

SDS-PAGE and Western blot analyses using antibodies for total cofilin and for cofilin phosphorylated at Ser3 (pS3) did not show significant difference between CTRL and LP-211 treated striatal or cortical neurons (**Figures 6B,D**). These results indicate that the shift of cofilin toward the acidic region observed in 2D-gels following stimulation of the 5-HT7R cannot be attributed to its phosphorylation at Ser3.

Activation of 5-HT7R Stimulates Axonal Outgrowth

Although neurite outgrowth is studied in cultured neurons before the occurrence of neuronal polarization, it is generally considered the equivalent of axonal extension (Conti et al., 1997). To determine whether 5-HT7R could indeed modulate axonal growth, we used microfluidic culture platforms that allow physical separation between soma and axons of cultured neurons (Park et al., 2006). Microfluidic chambers have been mostly used with HIPP neurons for their ability to develop long axons. Therefore, we first tested the effect of 2 h stimulation with LP-211 on neurite elongation of E18 mouse HIPP neurons cultured in traditional chamber slides. These cultures were immunostained with TuJ1 antibodies and counterstained with the nuclear marker DAPI, indicating that most of cultured cells were neurons (see panels in **Figure 7**). Consistent with previous results obtained with other 5-HT7R agonists (Kvachnina et al., 2005), we observed that the length of neurites significantly increased in LP-211-treated cultures

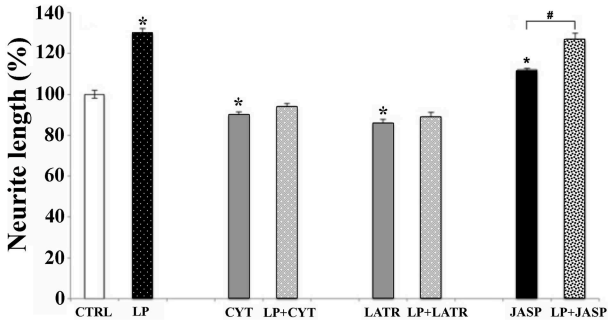

FIGURE 5 | Actin polymerization is required for neurite elongation induced by 5-HT7R stimulation. Striatal primary cultures were treated for 2 h with the 5-HT7R selective agonist LP-211 (LP, 100 nM). Cells were also treated with compounds depolymerizing actin filaments, as cytochalasin B (CYT, 100 nM) or latrunculin A (LATR, 2 μM), or promoting actin polymerization as jasplakinolide (JASP, 2 μM), with or without LP. The graph shows the neurite length expressed as percent values in reference to vehicle-treated cultures (CTRL, set to 100%). The bars represent means ± SEM from randomly selected fields for each cell culture condition ($n = 9$). Asterisk (*): values significantly different from CTRL by One Way ANOVA followed by Dunnett *post-hoc* test ($p < 0.05$). Number sign (#): values significantly different from each other by One Way ANOVA followed by Tuckey *post-hoc* test ($p < 0.05$).

compared to CTRL; the co-treatment of neurons with LP-211 and SB-269970 completely abolished neurite elongation, while addition of the antagonist alone did not affect neurite outgrowth (**Figure 7**).

Then, we cultured HIPP neurons in microfluidic chambers (Park et al., 2006), implementing culture medium with 100 nM of the agonist. Neurons were plated on one side of the culture chamber (soma compartment) and axons, but not dendrites, grew into the other compartment, passing through interconnecting micro-channels (450 μm long) (**Figure 8A**). We first verified whether in our system the only fibers crossing the channels were axons, by co-immunolabeling hippocampal neurons with Tuj1 (neuronal marker) and either anti-Tau (axon specific marker) or anti-Map2 (dendrite specific marker). As expected, the anti-Tau antibody labeled axons in both compartments, while dendritic labeling with Map2 was confined to the soma compartment (**Figure 8B**). Therefore, we plated HIPP neurons in the presence of either LP-211 or vehicle (CTRL), added to both compartments. Cultures were maintained for 6 days and axons reaching the appropriate compartment were counted daily, from 1DIV to 6DIV. In LP-211-treated cultures we observed a significant increase in the number of axons crossing the channels by 3 to 5DIV, clearly indicating that stimulation of the 5-HT7R promotes axonal growth, at least at early stages (**Figure 8C**). This difference disappeared at 6DIV, suggesting receptor desensitization, although we have also considered the hypothesis that the number of axons would eventually reach a plateau, since the number of channels to cross is limited. **Figure 8D** shows two typical cultures, grown in the presence of either DMSO or LP-211, indicating that, before plateau is reached, the number of axons reaching the side compartment is higher in the presence of 5-HT7R agonist.

Discussion

Neurotransmitters have a well-established role for neuronal communication in the adult CNS, but many of them, including serotonin, have been shown to act as signaling molecules during neuronal development regulating neurite outgrowth, dendritic spines shape and number, target selection and synapse formation (Van Kesteren and Spencer, 2003; Lesch and Waider, 2012).

Accordingly, a role for 5-HT7R in the regulation of neurite outgrowth was demonstrated on mouse hippocampal neurons *in vitro* (Kvachnina et al., 2005), and more recently on rat striatal and cortical cultures (Leo et al., 2009; Speranza et al., 2013). In line with these findings, in this paper we demonstrate that the neurite outgrowth observed in cultured embryonic neurons of mouse striatum, cortex and hippocampus is strongly stimulated by LP-211, a newly discovered highly selective 5-HT7R agonist (Hedlund et al., 2010). We show that this process requires the activation of the serine/threonine kinase mTOR, and the Rho GTPase Cdc42, in addition to Cdk5 activation and ERK phosphorylation, corroborating and extending our previous findings on rat neuronal cultures (Speranza et al., 2013).

In neuronal cells, mTOR is implicated in multiple processes, including transcription, ubiquitin-dependent proteolysis, and microtubule and actin dynamics, all of which are crucial for neuronal development and long-term modification of synaptic strength (Jaworski and Sheng, 2006). Moreover, mTOR is recruited following activation of various G protein-coupled receptors and thereby plays a role in synaptic plasticity through formation and maturation of new synapses (Hoeffer and Klann, 2010; Meffre et al., 2012). The two distinct multiprotein complexes formed by mTOR with different accessory proteins, mTORC1 and mTORC2, are differently sensitive to rapamycin (Takei and Nawa, 2014). Recent efforts have led to the development of torin 1, an ATP-competitive mTOR inhibitor blocking both mTORC1 and mTORC2 complex activity (Liu et al., 2010). Our results show for the first time that the neurite elongation induced by agonist stimulation of 5-HT7R is dependent on mTOR, as the outgrowth is completely inhibited by both rapamycin and torin 1. Accordingly, phosphorylation of the p70SK, one of the well known targets of mTOR (Laplante and Sabatini, 2012), was strongly increased by treatment of neuronal cultures with LP-211, and completely abolished by co-treatment with torin 1. These results are in agreement with the recent findings that the *in vivo* treatment with LP-211 stimulates mTOR signaling in the mouse brain (De Filippis et al., 2014) and highlight the key role of this pathway in mediating the effects induced by 5-HT7R stimulation in the CNS. On the other hand, our analyses indicate that phosphorylation of Akt, one of the well known targets of mTORC2 (Takei and Nawa, 2014), was not affected by the treatment of neuronal cultures with LP-211, suggesting that the intracellular cascade stimulated by 5-HT7R involves mainly mTORC1.

Another piece of the puzzle was provided by the recent studies in which Rho GTPases have been identified as key regulators of actin cytoskeleton rearrangement leading to axonal and dendritic growth. Thus, it has been suggested that Rho GTPases may act

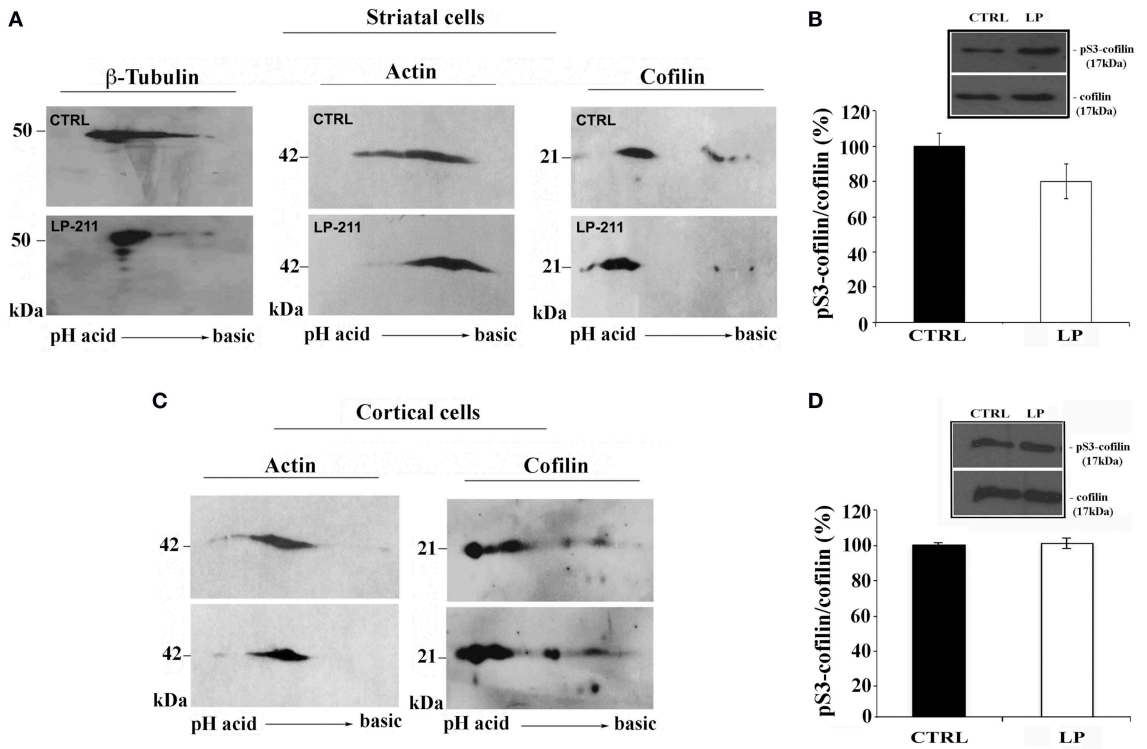

FIGURE 6 | Effect of LP-211 on expression levels of several cytoskeletal proteins in neuronal primary cultures. 2D immunoblot analyses, using antibodies against β-tubulin, actin or cofilin, were performed on **(A)** striatal cells or **(C)** cortical cells treated with vehicle (CTRL) or with the 5-HT7R selective agonist LP-211. The graphs on the right show the level of cofilin phosphorylation on Ser 3 (pS3-cofilin) in striatal **(B)** and cortical **(D)** neurons treated for 2 h with LP-211 (LP, 100 nM). The intensity of pS3-cofilin was normalized with that of total cofilin (means ± SEM; $n = 3$) and was expressed as percentage of values measured in the corresponding vehicle-treated cultures (CTRL, set to 100%). The insets display representative SDS-PAGE blots probed with antibodies against pS3-cofilin and cofilin. The molecular weights (kDa) are indicated on the right.

as a molecular switches integrating signals from the extracellular environment (Auer et al., 2011; Li et al., 2014). As a member of Rho GTPases protein family, Cdc42 is involved in neuronal morphogenesis including axon growth and guidance, dendritic spine plasticity and synapse formation (Luo, 2000). This GTPase is required for filopodia formation and for the concomitant activation of the actin regulator cofilin in growth cones (Garvalov et al., 2007). In accordance with these observations, our results clearly indicate that Cdc42 indeed plays a crucial role in the LP-211-dependent enhancement of neurite outgrowth. Thus, this Rho GTPase may be considered a key molecule in the signaling pathways coupling activation of 5-HT7R with the cytoskeleton reorganization.

As aforementioned, we previously demonstrated that ERK and Cdk5 are also activated during the LP-211-induced enhancement of neurite outgrowth (Speranza et al., 2013), which has been corroborated here in cultured neurons obtained from various regions of the mouse brain (Supplementary Figure 1). The involvement of ERK and Cdk5 are consistent with the well-known role of these pathways as critical signal transmission hubs positioned between a range of cell surface receptors and various cytoskeletal systems regulating neuronal morphology (Colucci-D'Amato et al., 2003; Jessberger et al., 2009). Thus, our new experimental evidence showing that mTOR and Cdc42 are also involved in the signaling pathways activated by 5-HT7R suggest that this newly found member of the 5-HT receptor family uses a variety of molecular tools to exert its effect on neurite outgrowth during development. Accordingly the link between some of these signal trasduction pathways has been demonstrated in several different experimental systems (Kvachnina et al., 2005; He et al., 2013). A plausible hierarchical order for these downstream 5-HT7R signaling components would envisage recruitment of Cdk5 and Cdc42, in analogy to recent findings by Duhr et al. (2014). These authors have shown that both these signaling pathways are involved in the 5-HT6R neurite elongation, and that Cdk5 constitutively interacts and phosphorylates the receptor. This cooperation might occur also for 5-HT7R, and Cdk5 would in turn activate ERK signaling, as observed in rat primary cultures (Speranza et al., 2013). ERK pathway might then activate mTORC1, as reviewed by Laplante and Sabatini (2012). In addition, Cdc42 could be a downstream Cdk5 mediator of neurite growth, operating in parallel to ERK pathway (Cheung et al., 2007).

The protruding force responsible for the extension of neuronal processes relies on the modulation of cytoskeleton dynamics, in particular on the regulation of microtubules and microfilaments

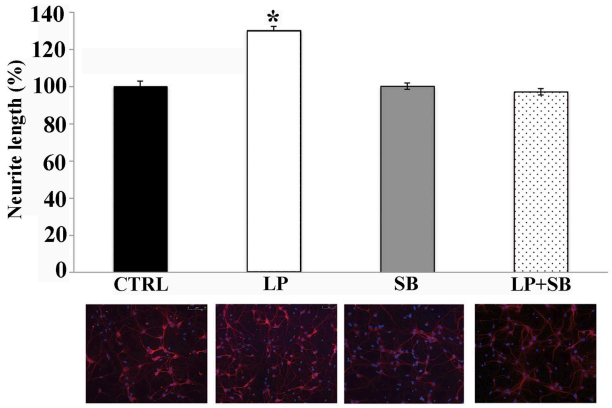

FIGURE 7 | Stimulation of 5-HT7R enhances neurite outgrowth in hippocampal primary cultures. Hippocampal cells were treated for 2 h with the 5-HT7R selective agonist LP-211 (LP, 100 nM), alone or in combination with the selective 5-HT7R antagonist SB269970 (SB, 100 nM). Neurite length was measured on cells stained with anti-Tuj1 antibody, and expressed as percentage of values measured in the corresponding vehicle-treated cultures (CTRL, set to 100%). The bars represent means ± SEM from randomly selected fields for each cell culture conditions ($n = 10$). Asterisk (*): values significantly different from CTRL by One Way ANOVA followed by Dunnett *post-hoc* test ($p < 0.05$). The panels below each bar display representative images of hippocampal neurons immunostained with the neuronal marker Tuj1 (red) and counterstained with the nuclear marker DAPI (blue; magnification 20x).

polymerization (Schaefer et al., 2008). Accordingly, our data indicate that the neurite outgrowth induced by 5-HT7R stimulation depends on actin filaments. Indeed the stimulating effect of LP-211 on neurite outgrowth was blocked by the addition of actin-binding drugs that promote net depolymerization of actin filaments, either by sequestering the free actin monomer pool (latrunculin) or by capping the fast-growing barbed ends of actin filaments (cytochalasin). On the other hand, the treatment with jasplakinolide, which stabilizes polymerization of actin filaments, increased the neurite length in the absence of LP-211. However, the co-treatment with jasplakinolide and LP-211 did not promote additional neurite outgrowth compared to the treatment with the agonist alone, suggesting that 5-HT7R activation may have stimulated the outgrowing mechanism at its maximum level. It is important to consider that the modulation of actin dynamics is an extremely complex process, as revealed by either stimulatory or inhibitory effects of actin polymerization drugs on axonal or dendritic growth (Bradke and Dotti, 1999; Gallo et al., 2002; Jones et al., 2004). These apparently divergent results may be attributed to the different stages of development/differentiation of neuronal processes, as well as the various experimental conditions (i.e., drug treatment, neuronal cultures) used by different investigators. Altogether, our data are consistent with the knowledge that the cellular mechanisms regulating neurite outgrowth are deeply influenced by actin dynamics (Schaefer et al., 2008).

Many extracellular signaling molecules enhancing neurite outgrowth exert profound effects on the organization of the actin cytoskeleton, mainly acting on actin-binding proteins, such as cofilin. The regulation of cofilin phosphorylation is a key convergence point of multiple cell signaling networks that link extracellular stimuli to actin cytoskeletal dynamics (Huang et al., 2006, 2013; Mizuno, 2013). It has been suggested that cofilin activity, modulated by a proper balance of its phosphorylation level, may act as a regulator for neurite extension in specific subcellular regions of the neuron (Endo et al., 2007; Figge et al., 2012). Interestingly, recent data indicate that stimulation of 5-HT7R is able to reverse the abnormal activation of cofilin in a mouse model of Rett syndrome (De Filippis et al., 2014). Here, following 5-HT7R agonist stimulation of both striatal and cortical neurons, we didn't detect significant changes of cofilin phosphorylation at Ser3 by Western blot analyses. On the other hand, the 2D gel profiles of cofilin protein from LP-211 treated cultures undergo significant changes compared to control cultures, showing a shift toward acidic pH of multiple spots without changes in molecular mass. These changes may be ascribed to phosphorylation on residues different from Ser3, or to other post-translational modifications. The detailed analyses of these modifications will be object of further investigation.

Many experiments using cultured embryonic hippocampal neurons have revealed that, as they develop, neurons initially generate several equivalent neurites, but then begin to polarize so that one neurite becomes an axon while the remaining neurites become dendrites (Dotti et al., 1988).

Interestingly, here we demonstrate for the first time that stimulation of the 5-HT7R enhances axonal elongation in cultured hippocampal neurons. These data, coupled with previous results indicating involvement of 5-HT7R in potentiating formation of dendritic spines in hippocampal neurons, strongly support the emerging role of 5-HT7R in shaping brain networks during development by modulating neuronal cytoarchitecture and connectivity (Kobe et al., 2012; Volpicelli et al., 2014).

In addition, the stimulating effect of 5-HT7R on axonal elongation open a new perspective suggesting the involvement of 5-HT7R in axonal pathfinding and regeneration, presumably through the activation of mTOR signaling and cofilin. Indeed, recent data indicate that mTOR pathways and cofilin activation play a key role in the mechanism underlying the ability of axon to regenerate (Stern et al., 2013; Lu et al., 2014).

Interestingly, the successful rescue of functional and behavioral deficits observed in animal models of Fragile X syndrome and Rett syndrome (Costa et al., 2012; De Filippis et al., 2014), following stimulation of the 5-HT7R, identify this receptor as a potential target for innovative pharmacological treatment of several neurodevelopmental diseases associated with abnormal CNS connectivity.

In summary, our results highlight the role of 5-HT7R and its signal transduction pathways in shaping neuronal morphology in embryonic forebrain neurons in culture, suggesting its involvement in the correct establishment of neuronal wiring during critical periods of CNS development. Similar mechanisms could operate also in the mature brain to modulate plasticity of neural circuits.

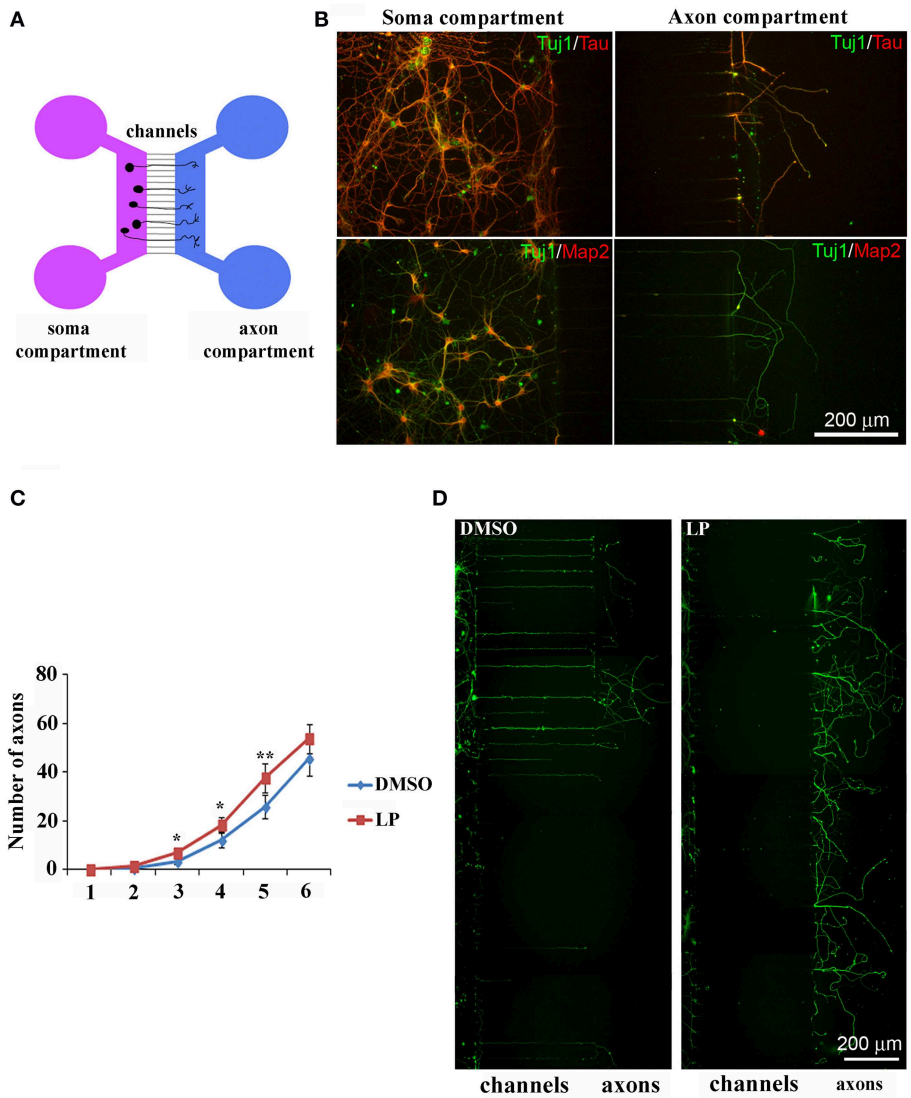

FIGURE 8 | Stimulation of 5-HT7R enhances axonal outgrowth in cultured hippocampal neurons. (A) Schematic representation of microfluidic chambers. **(B)** Co-immunolabeling of neurons cultured in microfluidic chambers with the neuronal marker Tuj1 (green) and either the axonal marker Tau (top row, red) or the dendritic marker Map2 (bottom row, red). Dendritic immunolabeling is confined in the soma compartment and only axons cross the micro-channels to reach the other compartment. **(C)** The number of axons crossing the micro-channels is significantly higher in LP-211 treated cultures (LP, red line), respect to control (DMSO, blue line), after 3, 4, and 5 DIV. Asterisk (*): values significantly different from DMSO by One Way ANOVA followed by Dunnett *post-hoc* test ($p < 0.05$). **(D)** Representative hippocampal cultures grown in the presence of DMSO or LP and immunolabeled with the Tuj1 antibody (green).

Author Contributions

LS, TG, MEDS, LL, AC, and EL performed experiments; FV, GCB, and ML analyzed data; UdP, MC, and CP-C designed research and wrote the paper.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnbeh.2015.00062/abstract

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5-HT7 receptor activation promotes an increase in TrkB receptor expression and phosphorylation

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The serotonin (5-HT) type 7 receptor is expressed throughout the CNS including the cortex and hippocampus. We have previously demonstrated that the application of 5-HT7 receptor agonists to primary hippocampal neurons and SH-SY5Y cells increases platelet-derived growth factor (PDGF) receptor expression and promotes neuroprotection against N-methyl-D-aspartate-(NMDA)-induced toxicity. The tropomyosin-related kinase B (TrkB) receptor is one of the receptors for brain-derived neurotrophic factor (BDNF) and is associated with neurodevelopmental and neuroprotective effects. Application of LP 12 to primary cerebral cortical cultures, SH-SY5Y cells, as well as the retinal ganglion cell line, RGC-5, increased both the expression of full length TrkB as well as its basal phosphorylation state at tyrosine 816. The increase in TrkB expression and phosphorylation was observed as early as 30 min after 5-HT7 receptor activation. In addition to full-length TrkB, kinase domain-deficient forms may be expressed and act as dominant-negative proteins toward the full length receptor. We have identified distinct patterns of TrkB isoform expression across our cell lines and cortical cultures. Although TrkB receptor expression is regulated by cyclic AMP and Gαs-coupled GPCRs in several systems, we demonstrate that, depending on the model system, pathways downstream of both Gαs and Gα12 are involved in the regulation of TrkB expression by 5-HT7 receptors. Given the number of psychiatric and degenerative diseases associated with TrkB/BDNF deficiency and the current interest in developing 5-HT7 receptor ligands as pharmaceuticals, identifying signaling relationships between these two receptors will aid in our understanding of the potential therapeutic effects of 5-HT7 receptor ligands.

Keywords: 5-HT7, TrkB, transactivation, phosphorylation, protein expression

INTRODUCTION

Neurotrophins, or neurotrophic factors, include nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Skaper, 2012). One of the receptors for BDNF is the tropomyosin-related kinase B (TrkB) receptor (Schecterson and Bothwell, 2010). TrkB receptors are transmembrane receptor tyrosine kinases (RTKs) found in dendritic spines, axons and neuronal cell bodies. There are three TrkB isoforms, full-length TrkB (TrkB-FL), TrkB-Shc, and TrkB-T1, that all have the same extracellular ligand-binding domain and transmembrane domain, however, the T-Shc and TrkB-T1 receptors are truncated and lack tyrosine kinase activity found in TrkB-FL (Fenner, 2012). Both TrkB-T1 and TrkB-T-Shc receptors are thought to modulate TrkB-FL activity by forming heterodimers with TrkB-FL receptors as well as by competing for and sequestering BDNF (Fenner, 2012; Gomes et al., 2012; Wong and Garner, 2012). TrkB-FL receptors can be phosphorylated at specific tyrosine residues including tyrosine 816 (Y816), the site for activating phospholipase Cγ (Huang and Reichardt, 2003). Recent work suggests that the relative number of TrkB isoforms expressed (e.g., TrkB-FL vs. TrkB-T1) is key for the neuroprotective signaling of BDNF. For example, increases in TrkB-T1 (via increased transcription, not as a degradation product of the full-length receptor) decrease TrkB-FL activity after excitotoxicity (Gomes et al., 2012).

A common theme in the regulation of TrkB signaling involves the second messenger cyclic AMP. BDNF-induced TrkB phosphorylation is regulated by cyclic AMP signaling in hippocampal neurons (Ji et al., 2005) and TrkB cell surface expression is regulated by cyclic AMP in retinal ganglion cells (RGCs) (Meyer-Franke et al., 1998). TrkB expression is also regulated by cyclic AMP signaling in several other systems (Heo et al., 2013). For example, the activation of the Gαs-coupled adenosine 2A receptors increases

Abbreviations: 5-HT, serotonin; BDNF, brain-derived neurotrophic factor; cyclic AMP, cyclic adenosine mono-phosphate; GPCR, G protein-coupled receptor; PDGF, platelet-derived growth factor; pLTF, phrenic long-term facilitation; PMF, phrenic motor facilitation; RGC, retinal ganglion cell; RTK, receptor tyrosine kinase; TrkB, tropomyosin-related kinase B.

TrkB expression in the spinal cord (Golder et al., 2008). A2A receptors also promote an increased expression and release of the BDNF in rat cortical cultures (Jeon et al., 2011). In addition to changes in protein expression, GPCRs can increase RTK activation via a process called transactivation: adenosine 2A receptors and the Gαs-coupled dopamine D1 receptor are both able to transactivate TrkB (Lee et al., 2002; Wiese et al., 2007; Iwakura et al., 2008). 5-HT7 receptors are also reported to couple to Gαs (Bard et al., 1993; Lovenberg et al., 1993; Ruat et al., 1993) as well as Gα12 (Kvachnina et al., 2005).

TrkB-FL has been linked to neuroprotective effects after CNS insults by excitotoxicity, amyloid-β, and the HIV protein gp120 (Almeida et al., 2005; Nosheny et al., 2005; Kitiyanant et al., 2012) and changes in TrkB-FL are associated with neurodegenerative diseases (Longo et al., 2007; Zuccato and Cattaneo, 2009). In schizophrenia, as our understanding of the condition has shifted to viewing the disease as a neurodevelopmental illness, there has been an increased focus on neurotrophic factor signaling in its pathophysiology (Kalkman, 2009) and there is a strong association with BDNF with valine at position 66 (instead of methionine) and the illness (Rybakowski, 2008). Decreases in BDNF signaling are also associated with glaucoma and retinal degeneration (Gupta et al., 2014). A direct physiological link between 5-HT7 receptors and TrkB expression has been observed in a model of phrenic long-term facilitation (pLTF), a form of plasticity in the respiratory system that occurs as a response to hypoxia (Mitchell and Johnson, 2003). This centrally-mediated form of potentiation is associated with changes in 5-HT, BDNF, and TrkB signaling (Fuller et al., 2001; Baker-Herman et al., 2004). Recently, Hoffman and Mitchell demonstrated that spinal 5-HT7 receptor activation by the 5-HT7 receptor agonist, AS-19, was able to induce phrenic motor facilitation (PMF) that was dependent on new TrkB synthesis and activation (Hoffman and Mitchell, 2011). Initially these authors proposed a model whereby 5-HT7 receptor agonists might promote pLTF after hypoxia; however subsequent findings suggested the opposite: 5-HT7 agonists countered pLTF and antagonists (SB-269970) facilitated pLTF in conjunction with 5-HT2 receptor activation (Hoffman and Mitchell, 2013).

We have recently reported that long-term (24 h) application of 5-HT7 agonists (5-carboxamidotryptamine (5-CT) and LP 12) increases the expression of the platelet-derived growth factor (PDGF) β receptor in a PKA-dependent manner (Vasefi et al., 2012) and this increase in PDGF receptor expression was sufficient to protect primary hippocampal neurons against NMDA-induced excitotoxicity (Vasefi et al., 2013). Given that TrkB expression is regulated by several other Gαs-coupled receptors, we examined the ability of 5-HT7 agonists and antagonists to regulate TrkB receptor isoform expression and phosphorylation in primary mouse cerebral cortical cultures, the human neuroblastoma-derived SH-SY5Y cell line, and the RGC line 5 (RGC-5).

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

LP12 [4-(2-Diphenyl)-*N*-(1,2,3,4-tetrahydronaphthalen-1-yl)-1-piperazinehexanamide hydrochloride] and H89 were purchased from Sigma-Aldrich (St. Louis, MO). SB 258719 ((R)-3,N-Dimethyl-N-[1-methyl-3-(4-methylpiperidin-1-yl)propyl]benzene sulfonamide) and SB 269970 (R-3-(2-(2-(4-methylpiperidin-1-yl)ethyl)-pyrrolidine-1-sulfonyl)-phenol were purchased from Tocris (Ellisville, MO, USA). Rhosin was purchased from Calbiochem (Billerica, MA). Antibodies against TrkB (catalog #sc-8316, used at a dilution of 1:500) and β-actin (catalog #sc-81178, used at a dilution of 1:500) were purchased from Santa Cruz (Santa Cruz, CA). The TrkB-Y816 antibody (catalog #ab75173, used at a dilution of 1:500) was purchased from Abcam (Toronto, ON). The secondary antibodies [horseradish peroxidase (HRP) enzyme-conjugated mouse polyclonal IgG and HRP enzyme conjugated rabbit polyclonal IgG] were both purchased from Thermo Fisher (Pittsburgh, PA).

PRIMARY CEREBRAL CORTICAL CULTURES

E17 to E19 mouse embryos were removed from pregnant CD-1 mice (Harlan, Indianapolis, IN) and transferred to chilled dissection media (15 mL HEPES buffer in 500 mL HBSS with 2.5 g glucose (0.6%), 10 g sucrose (2%), pH 7.4, final osmolality 320–335). The cortex was removed, separated, and trypsinized with 0.25% trypsin for 20 min at 37°C. Cells were then plated on poly-d-lysine-coated culture dished and grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were plated with plating media (DMEM, supplemented with 10% fetal bovine serum, 10% horse serum) for the first 2–4 h until attached and then with feeding media consisting of Neurobasal medium and B-27 supplement (Life Technologies, Burlington, ON) without serum. Drug treatments were performed 7–10 days after plating the cells. Media was changed twice per week. To stop the overgrowth of non-neuronal cells, a mitotic inhibitor (81 μM 5-fluoro-2′-deoxyuridine and 200 μM uridine added to media) was added for 24 h once cells reached confluency.

CELL CULTURE

SH-SY5Y cells were grown on DMEM with Ham's F12 in a 1:1 ratio (Thermo Fisher), 10% fetal bovine serum and 1% penicillin/streptomycin. Media was changed every 2–3 days and cells were kept at 37°C and 5% CO_2. Cells were serum and antibiotic deprived 24 h prior to drug treatment to prevent any interactions with growth factors. The RGC-5 cell line was grown on Dulbecco's Modified Eagle Medium (DMEM) with low glucose (Thermo Fisher), 10% horse serum and 1% penicillin/streptomycin. Media was changed every 2 days and cells were kept at 37°C and 5% CO_2.

WESTERN BLOT

After drug treatment, cells were washed with phosphate-buffered saline (PBS) and lysed in chilled lysis buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 30 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 1% Triton X-100; supplemented with Halt Protease and Phosphatase Inhibitor (Thermo, Fisher, Markham, Ontario) prior to use). Cells were scraped, sheared using 26 gage needles, and centrifuged at 14,000 × g for 20 min at 4°C and the supernatant was collected. Protein concentrations were determined using a BCA protein assay protocol (Thermo Fisher). Homogenates were subjected to SDS-PAGE and proteins were

transferred to nitrocellulose membranes, blocked with 5% non-fat dry milk in Tris-buffered saline and 0.1% Tween-20 for 1 h at room temperature or overnight at 4°C, and incubated in primary antibodies for 1 h at room temperature or overnight at 4°C. Membranes were washed three times in Tris-buffered saline with 0.1% Tween-20, incubated with HRP-conjugated secondary antibodies for 1 h at room temperature, washed again, and bound antibodies were visualized by enhanced chemiluminescence using Luminata Crescendo substrate (Millipore, Etobicoke, Ontario). Images of Western blots were taken on a Kodak 4000MM Pro Imaging Station, and densitometric analyses were performed using Kodak Molecular Imaging software. Protein bands were then identified by their molecular weights: TrkB-FL and TrkB-Y816 at 145 kDa, TrkB T1 at 95 kDa, TrkB T-Shc at 105 kDa, and bands were normalized to the loading control, β-actin.

STATISTICAL ANALYSIS

Mean protein expression/phosphorylation and standard errors were calculated using Microsoft Excel. GraphPad Prism® software was used for graphing and for analyzing statistical significance using a One-Way ANOVA with a Dunnett's or Bonferroni's post-test. Significance level was set to $\alpha = 0.05$.

ANIMALS

All animal experiments were performed in agreement with the guidelines of the policies on the Use of Animals at the University of Waterloo under animal utilization project proposal (AUPP) #13-24 in accordance with standards of the Canadian Council on Animal Care.

RESULTS

24 h ACTIVATION OF 5-HT7 RECEPTORS INCREASE TrkB EXPRESSION

Based on our previous findings that 24 h treatment of SH-SY5Y cells and primary cultures with 5-HT7 receptor agonists increased the expression of the PDGFβ receptor, we incubated primary mouse cerebral cortical cultures with the 5-HT7 receptor agonist LP 12 (300 nM) in the absence or presence of the 5-HT7 receptor antagonist SB 258719 (1 μM). LP 12 increased the expression of full-length TrkB (TrkB-FL) and this was blocked by the antagonist (**Figure 1A**). The same treatments resulted in similar effects in SH-SY5Y cells (**Figure 1B**). In both cell types, LP 12-induced increases in TrkB were also blocked using the inverse 5-HT7 receptor agonist, SB 269970 (data not shown). The concentration of 300 nM LP 12 was chosen based on our previous work on the regulation of PDGFβ receptor expression in similar model systems (Vasefi et al., 2013). Preliminary experiments in SH-SY5Y cells demonstrated that the application of concentrations higher than 300 nM resulted in sub-maximal increases in TrkB receptor expression (data not shown). In addition to TrkB-FL, we detected the expression of TrkB-T1 at 95 kDa and TrkB-T-Shc at 105 kDa. In both cortical neurons and SH-SY5Y cells, LP 12 treatment also resulted in an increase in TrkB-T-Shc and T1 by approximately 1.5 fold however these changes were not significant. In RGC-5 cells, 24 h LP 12 treatment significantly increased TrkB-FL and TrkB-T-Shc and there was a trend toward an even larger increase in TrkB-T1 but the result was much more variable and not statistically significant (**Figure 1C**). In all three model systems, the phosphorylation of Y816 on the full length TrkB receptor (a phosphorylation site associated with increased TrkB receptor activity; Huang and Reichardt, 2003) was significantly increased over baseline (1.40 ± 0.15, $n = 7$ in cortical neurons, 1.34 ± 0.12, $n = 7$ in SH-SY5Y cells, and 1.31 ± 0.12, $n = 7$ in RGC-5 cells).

2 h ACTIVATION OF 5-HT7 RECEPTORS INCREASES TrkB EXPRESSION

We then investigated whether a shorter incubation of cortical neurons, SH-SY5Y or RGC-5 cells with LP 12 would result in similar changes in TrkB expression and phosphorylation. In cortical cultures, 2 h treatment with LP 12 failed to alter TrkB receptor expression (**Figure 2A**). Similarly, 2 h incubation of *ex vivo* hippocampal slices resulted in variable changes in TrkB expression, however this treatment significantly increased TrkB Y816 phosphorylation (1.24 ± 0.06 fold vs. vehicle, $n = 3$, $p < 0.05$, unpaired *t*-test, data not shown). In SH-SY5Y and RGC-5 cells, LP 12 treatment increased the expression of TrkB-FL but differentially increased TrkB-T1 in SH-SY5Y cells and T-Shc in RGC-5 cells (**Figures 2B,C**). A significant increase in Y816 phosphorylation was observed in RGC-5 cells (1.43 ± 0.09, $n = 4$) but not SH-SY5Y cells (1.22 ± 0.08, $n = 6$) and cortical neurons (1.59 ± 0.39, $n = 5$) after 2 h LP 12 treatment.

BOTH Gαs AND Gα12 PATHWAYS ARE INVOLVED IN 5-HT7 RECEPTOR-INDUCED TrkB EXPRESSION

There is evidence that 5-HT7 receptors couple to both Gαs and Gα12 (Bard et al., 1993; Lovenberg et al., 1993; Ruat et al., 1993; Kvachnina et al., 2005). To determine which G protein pathway(s) leads to an increase in TrkB expression we incubated SH-SY5Y and RGC-5 cells with the cyclic AMP-dependent protein kinase (PKA) inhibitor, H89, or the RhoA inhibitor, rhosin. In SH-SY5Y, pretreatment with H89 (30 min, 10 μM) attenuated the increase in TrkB (FL) by LP 12 (**Figure 3A**). However, the RhoA inhibitor, rhosin (30 min, 30 μM) also inhibited the increase in TrkB expression (**Figure 3B**). In contrast, rhosin, but not H89, blocked LP 12-induced increases in TrkB expression in RGC-5 cells (**Figures 3C,D**). Taken together these results suggest that multiple G protein pathways may be involved in promoting TrkB expression downstream of the 5-HT7 receptor and that different pathways may be involved in different systems.

LP 12 TREATMENT RESULTS IN DIFFERENT PATTERNS OF TrkB ISOFORM EXPRESSION IN SH-SY5Y AND RGC-5 CELLS

We performed a time course experiment with LP 12 in the two models, SH-SY5Y and RGC-5 cells, that displayed differences in TrkB expression at 2 h. Both cell types were treated for 0.5, 1, 1.5, 2, 3, or 4 h with 300 nM LP 12 and changes in the three TrkB receptor isoforms were measured. As shown in **Figures 4A,B**, we observed very rapid changes in TrkB isoform expression as well as different patterns of TrkB isoform expression. In SH-SY5Y cells, changes were observed with all isoforms (however only changes in TrkB-FL at 1.5 and 2 h and TrkB-T1 at 2 h were significant, **Figure 4A**). In RGC-5 cells, changes in TrkB-FL were observed at earlier time points and LP 12 treatment did not significantly affect the expression of the other isoforms (**Figure 4B**). Note that

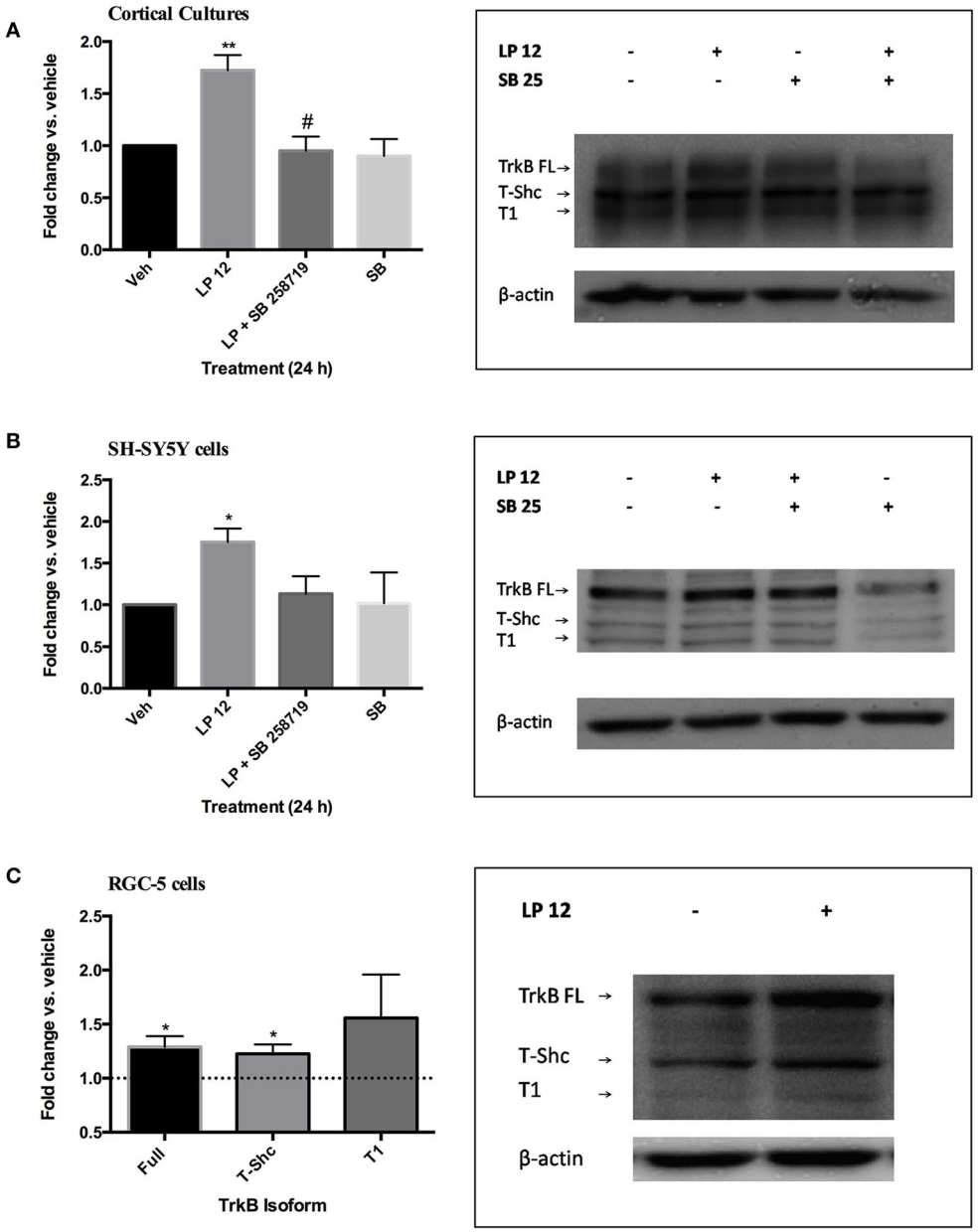

FIGURE 1 | 5-HT7 receptor activation increases TrkB receptor expression in several model systems. (A) Primary mouse cerebral cortical cultures were treated for 24 h with vehicle, 300 nM LP 12, 1 μM SB 258719, or both. The expression of TrkB-FL was normalized to β-actin and compared to the untreated sample (vehicle). $^{**}p < 0.01$ compared to vehicle, #, $p < 0.05$ compared to LP 12, $n = 5$, ANOVA analysis with Bonferroni's post-test. Representative western blots are shown for all TrkB isoforms and β-actin. Note that for "A" only the order of samples in the blot is different from the graph. **(B)** SH-SY5Y cells were treated as in "A." $^{*}p < 0.05$, $n = 10$. **(C)** RGC-5 cells were treated with vehicle or 300 nM LP 12 for 24 h. The expression of TrkB-FL (145 kDa), TrkB-T-Shc (105 kDa) and TrkB-T1 (95 kDa) were normalized to β-actin and compared to vehicle. $^{*}p < 0.05$, $n = 7$–9, one-sample *t*-test. Representative blots are shown for all TrkB isoforms and β-actin.

unlike **Figure 2C**, in these five time-course experiments we did not observe changes in TrkB-T-Shc levels.

5-HT7 RECEPTOR-INDUCED TrkB RECEPTOR TRANSACTIVATION

In addition to being activated by its ligand, BDNF, TrkB receptors can be transactivated after activation of GPCRs including adenosine 2A and dopamine D1 receptors (Lee et al., 2002; Wiese et al., 2007; Iwakura et al., 2008). To determine if 5-HT7 receptors are able to acutely transactivate TrkB receptors we performed a time-course similar to that described in **Figure 4** but with the addition of a 15 min time point. Interestingly, in SH-SY5Y cells, TrkB receptor transactivation was very brief (15 min) whereas in RGC-5 cells, the transactivation time course was more typical of that reported by others; the increase in TrkB

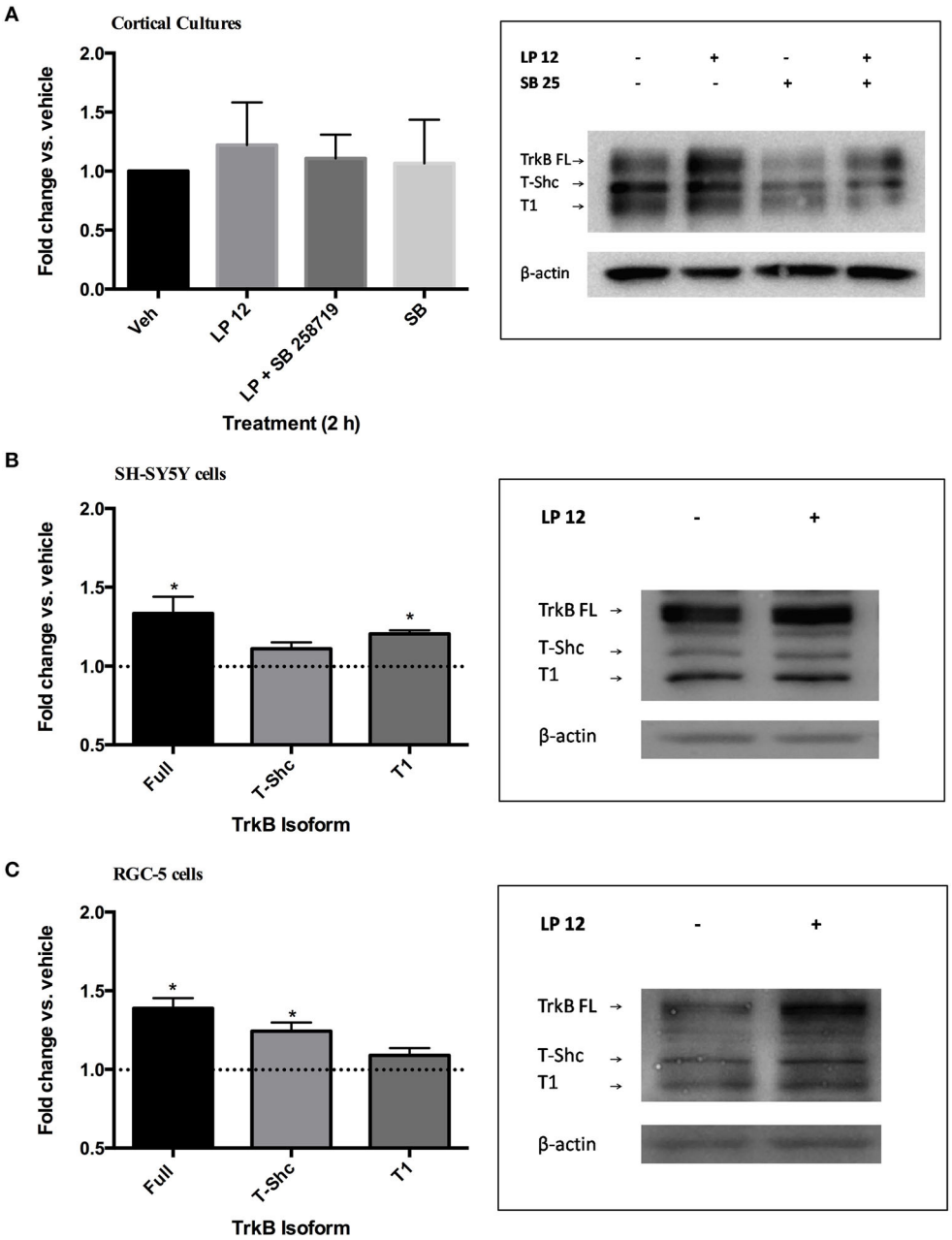

FIGURE 2 | Short-term incubation with LP 12 differentially alters TrkB isoform expression. (A) Cortical cultures were treated as in **Figure 1A** for 2 h. The expression of TrkB-FL was normalized to β-actin and compared to the untreated sample (vehicle). $n = 5$. Representative blots are shown for each TrkB isoform and β-actin. **(B)** SH-SY5Y cells were treated for 2 h with 300 nM LP 12. Expression of TrkB-FL, T-Shc, and T1 were normalized to β-actin and compared to vehicle. $*p < 0.05$, $n = 3$, one-sample *t*-test. Representative blots are shown for all TrkB isoforms and β-actin. **(C)** RGC-5 cells were treated as in "B." $n = 3$–6.

receptor phosphorylation after LP 12 treatment lasted up to 2 h (**Figure 5**).

DISCUSSION

In cortical cultures, SH-SY5Y cells, and RGC-5 cells, 24 h LP 12 treatment increased the expression of full length TrkB expression and its basal phosphorylation at Y816 and although the expression of T-Shc and T1 were also elevated over baseline in all models, only TrkB-T-Shc was significantly increased in RGC-5 cells. Over a shorter time period, differences in 5-HT7 receptor regulation of TrkB receptor isoforms were observed. In SH-SY5Y cells, 2 h LP 12 treatment increased TrkB-FL and TrkB-T1 isoforms, whereas TrkB-FL and TrkB-T-Shc were increased in RGC-5 cells. TrkB-T1 and T-Shc are generally ascribed dominant-negative activity toward full length TrkB (Gomes et al., 2012; Wong and Garner, 2012) as well as regulating TrkB cell surface

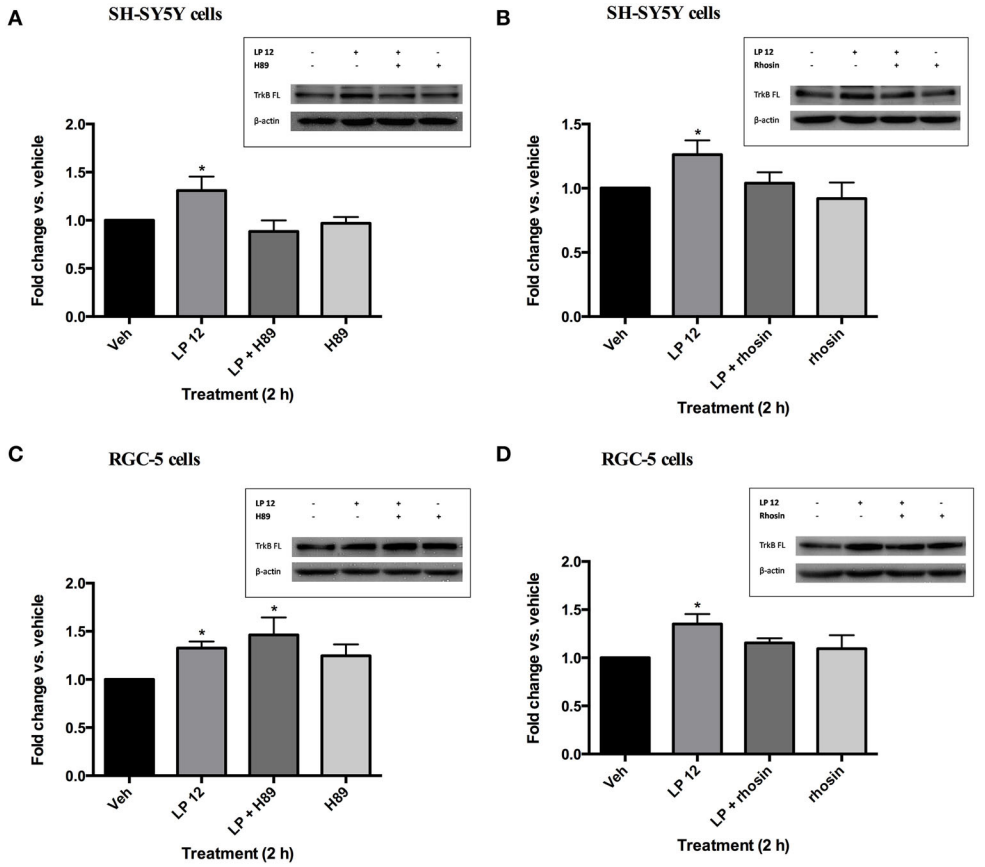

FIGURE 3 | Different pathways are involved in 5-HT7 receptor-induced increases in TrkB expression in SH-SY5Y cells and RGC-5 cells. SH-SY5Y cells were pre-treated with 10 μM H89 **(A)** or 30 μM rhosin **(B)** for 30 min prior to the addition of 300 nM LP 12 for 2 additional h. RGC-5 cells were similarly treated with H89 **(C)** or rhosin **(D)** prior to incubation with LP 12. The expression of TrkB-FL was normalized to β-actin and compared to the untreated sample (vehicle). $*p < 0.05$ compared to vehicle, $n = 4$ **(A–C)**, $n = 5$ **(D)**, ANOVA analysis with Dunnett's post-test.

expression (Haapasalo et al., 2002). Thus, signaling pathways that regulate TrkB expression and/or cell surface localization will produce a complex net effect of changes in TrkB-FL and the dominant negative forms. In neurons, this complexity is compounded by the relative localization of TrkB forms at synapses, cell bodies, etc. Although we observed changes in TrkB isoform expression in cortical cultures at 24 h, we did not see reliable changes over shorter time periods. This may be due to the reduced sensitivity of measuring changes in mixed neuronal cultures or the relatively lower expression of 5-HT7 receptors in the cortex compared to other brain regions (Horisawa et al., 2013).

Based on our previous work (Vasefi et al., 2012), and the literature identifying Gαs-coupled receptors and cyclic AMP pathways in regulating TrkB receptor expression (Ji et al., 2005; Heo et al., 2013), we predicted that 5-HT7 receptor activation would increase TrkB receptor levels via the Gαs-cyclic AMP-PKA pathway. However, 5-HT7 receptors are also reported to couple to Gα12 and Rho GTPase pathways, specifically the activation of RhoA and cdc42 (Kvachnina et al., 2005). As both of these monomeric G proteins are involved in cell growth in neurons, the Ponimaskin group recently demonstrated the involvement of 5-HT7 receptor/Gα12 in dendritic growth and synaptic activity/plasticity (Kobe et al., 2012). In our model systems, the changes in TrkB expression involved PKA and RhoA signaling pathways, or both, depending on the cell line. Many of the effects of 5-HT7 receptor signaling observed by Kobe et al. were in developing neurons after a 4 days treatment with the 5-HT7 (and 5-HT1a) agonist, 5-CT (Kobe et al., 2012). We observed changes in TrkB expression after 24 h as well as over shorter time periods. Interestingly, the TrkB receptor and BDNF signaling are intimately involved with many of the processes Kobe et al. observed in their systems (Kobe et al., 2012): see recent reviews on the involvement of TrkB/BDNF in dendritic spine formation (Bennett and Lagopoulos, 2014), synaptogenesis (Luikart and Parada, 2006), and synaptic plasticity (Lu et al., 2014).

The term transactivation is used to describe acute and usually very brief (5–15 min) GPCR-induced activation of RTKs that does not involve changes in RTK protein expression. This may occur through entirely intracellular signal transduction pathways, as in the case of 5-HT-induced transactivation of the PDGFβ and TrkB receptor in SH-SY5Y cells (Kruk et al., 2013). Another mechanism involves a GPCR-induced metalloproteinase-dependent shedding of growth factor ligand (such as EGF) to activate its RTK (Wetzker and Bohmer, 2003). TrkB receptor transactivation often differs

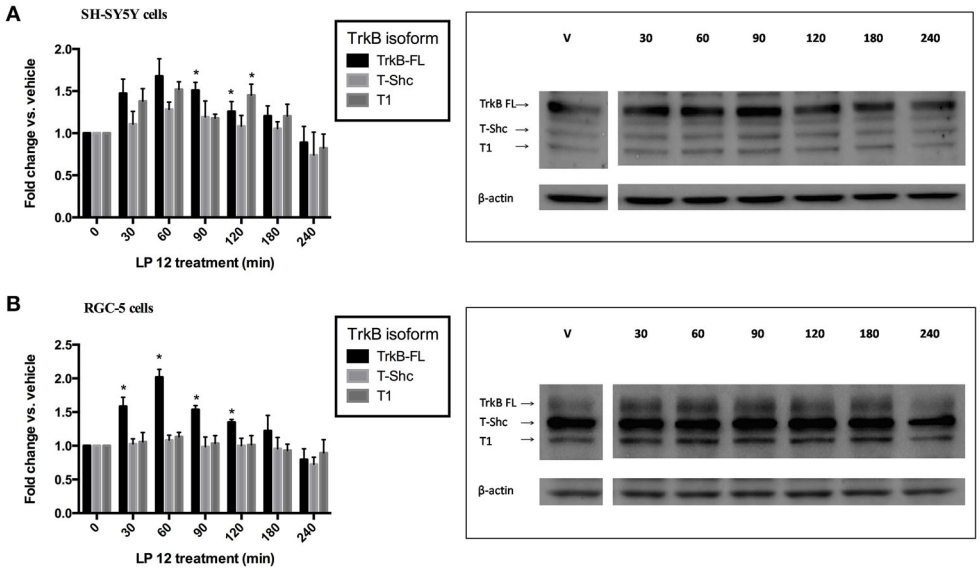

FIGURE 4 | Time course for the effects of LP 12 on TrkB isoform expression. SH-SY5Y cells **(A)** or RGC-5 cells **(B)** were incubated for 30–240 min with 300 nM LP 12. Expression of TrkB-FL, T-Shc, and T1 were normalized to β-actin and compared to vehicle. $^*p < 0.05$, $n = 3$–4 (SH-SY5Y), $n = 5$–6 (RGC-5 cells), ANOVA with Dunnett's post-test. Representative blots are shown for all TrkB isoforms and β-actin.

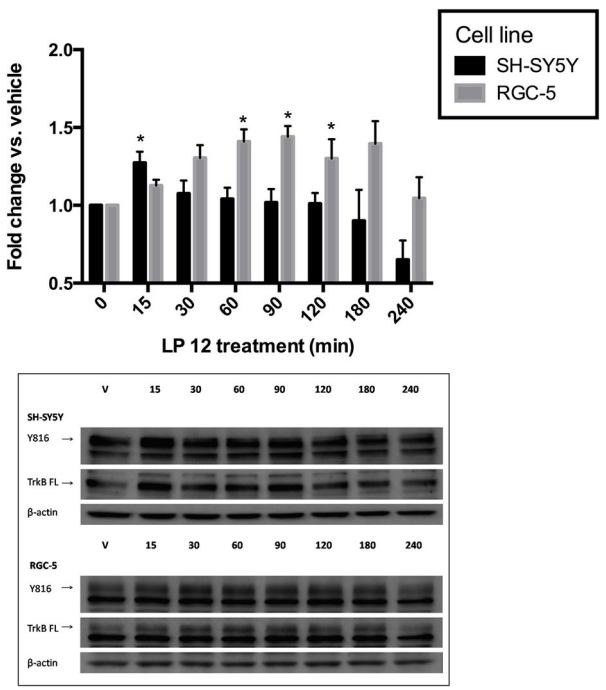

FIGURE 5 | 5-HT7 receptor transactivation of the TrkB receptor. SH-SY5Y cells (black bars, upper three blots) or RGC-5 cells (gray bars, lower three blots) were incubated for 15–240 min with 300 nM LP 12. Phosphorylation of the TrkB was measured using an anti-TrkB-phospho-Y816 antibody and compared to vehicle. $^*p < 0.05$, $n = 4$ (SH-SY5Y), $n = 5$ (RGC-5 cells), ANOVA analysis with Dunnett's post-test. The data from each cell line were collected independently thus the statistical test for each cell line were analyzed independently, however the data are presented together for comparison purposes. Representative blots are shown for phosphor-Y816, TrkB isoforms, and β-actin.

from the transactivation of other RTKs in that it typically manifests as a longer-lasting increase in receptor phosphorylation (Lee et al., 2002). Interestingly, we observed three distinct patterns of TrkB receptor transactivation by LP 12 in the three systems we investigated. Transactivation of TrkB was not reliably observed in cortical cultures. In SH-SY5Y, we observed a brief (15 min) transactivation that quickly returned to baseline, typical of many other RTK transactivation pathways. In RGC-5 we observed a transactivation typical of the TrkB receptor: one that was delayed in onset but much longer lasting. It remains to be determined what factors contribute to TrkB transactivation differing kinetically from the transactivation of other RTKs.

Given the interest in targeting growth and neurotrophic factor signaling pathways in CNS disease, an attempt to develop direct small molecule modulators for RTKs are ongoing, and in fact the TrkB receptor has been at the forefront of this avenue of investigation (Longo et al., 2007; Webster and Pirrung, 2008). An alternative approach to modulating RTKs in the CNS is to identify GPCRs linked to RTK signaling and expression. For 5-HT7 receptors, we have demonstrated such an approach *in vitro*: the 5-HT7 receptor ligand, LP 12, increases PDGFβ receptor expression in hippocampal neurons to provide neuroprotection against excitotoxicity (Vasefi et al., 2013). The development of selective 5-HT7 receptors able to cross the blood-brain barrier (Monti et al., 2014) will allow us to test the ability of 5-HT7 receptor ligands to modify RTK activity *in situ* in the CNS. Glaucoma is a family of eye diseases that results in visual field loss and eventual blindness (Weinreb et al., 2014). A reduction in BDNF is thought to contribute to the complex mechanisms of RGC death in glaucoma (Gupta et al., 2014). In rat, application of exogenous BDNF to the retina through intravitreal injection resulted in a promotion of RGC survival (Ko et al., 2001) thus there is extensive interest in delivering exogenous BDNF to the retina

as a glaucoma therapy. This has resulted in several approaches directed toward the delivery of neuroprotective genes to the retina in a less-invasive manner (Park et al., 2012; Alqawlaq et al., 2014). An alternative approach would be to exploit the 5-HT7 receptor's ability to promote TrkB expression and phosphorylation *in situ* in the retina. We began to explore this possibility *in vitro* using the RGC-5 cell line. Unfortunately, the integrity of the RGC-5 cell line has recently been compromised given investigations that revealed that the cell line does not express ubiquitous markers of RGCs and that its origin is from mouse and not rat as previously reported (Van Bergen et al., 2009). Nevertheless, 5-HT7-TrkB receptor linkages have been demonstrated here in two cell lines as well as in cortical cultures, and is likely present in the spinal cord (Hoffman and Mitchell, 2011, 2013). Therefore, further investigation of the possibility of 5-HT7 receptor-induced regulation in TrkB signaling in the retina and throughout the CNS is warranted.

AUTHOR CONTRIBUTIONS

Anshula Samarajeewa contributed most of the SH-SY5Y and RGC-5 data. Lolita Goldemann contributed much of the 24 h data in SH-SY5Y cells. Maryam S. Vasefi mentored several of the contributors as well as being involved in data collection. Nawaz Ahmed and Nyasha Gondora produced the data from cortical neurons. Chandni Khanderia collected RGC-5 data. John Mielke is the co-supervisor for Nyasha Gondora. Michael A. Beazely is the supervisor for undergraduate students Anshula Samarajeewa, Lolita Goldemann, and Chandni Khanderia as well as graduate students Maryam S. Vasefi, Nawaz Ahmed, and Nyasha Gondora. Anshula Samarajeewa, Maryam S. Vasefi, Nawaz Ahmed, Nyasha Gondora, and Michael A. Beazely wrote and edited the manuscript.

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Differential responses to acute administration of a new 5-HT7-R agonist as a function of adolescent pre-treatment: phMRI and immuno-histochemical study

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LP-211 is a new, selective agonist of serotonin (5-hydroxytryptamine, 5-HT) receptor 7 (5-HT7-R), which is part of a neuro-transmission system with a proposed role in neural plasticity and in mood, cognitive and sleep regulation. Adolescent subchronic LP-211 treatment produces some persisting changes in rats' forebrain structural and functional parameters. Here, using pharmacological MRI (phMRI), we investigated the effect of acute administration with LP-211 (10 mg/kg i.p.), or vehicle, to adult rats previously exposed to the same drug (0.25 mg/kg/day for 5 days), or vehicle, during adolescence (44–48 post-natal days); histology and immuno-histochemistry were performed *ex vivo* to evaluate neuro-anatomical and physiological long-term adaptation to pharmacological pre-treatment. The phMRI signal reveals forebrain areas (i.e., hippocampus, orbital prefrontal cortex), activated in response to LP-211 challenge independently of adolescent pre-treatment. In septum and nucleus accumbens, sensitized activation was found in adolescent pre-treated rats but not in vehicle-exposed controls. Immuno-histochemical analyses showed marked differences in septum as long-term consequence of the adolescent pre-treatment: increased level of 5-HT7-R, increased number of 5-HT7-R positive cells, and enhanced astrocyte activation. For nucleus accumbens, immuno-histochemical analyses did not reveal any difference between adolescent pre-treated rats and vehicle-exposed controls. In conclusion, subchronic LP-211 administration during adolescence is able to induce persistent physiological changes in the septal 5-HT7-R expression and astrocyte response that can still be observed in adulthood. Data shed new insights into roles of 5-HT7-R for normal and pathologic behavioral regulations.

Keywords: hippocampus, nucleus accumbens, septum, limbic/cortical loop, LP-211, Ph-MRI

INTRODUCTION

The serotonin (5-hydroxytryptamine, 5-HT) receptor 7 (5-HT7-R), the most recently identified member in the family of G-protein-coupled serotonin receptors, is characterized by a widespread expression in the central nervous system, in the peripheral nervous system and in the periphery (Ullmer et al., 1995; Pierce et al., 1997; Nilsson et al., 1999; Neumaiera et al., 2001; Meuser et al., 2002). The topography of 5-HT7-R distribution in the brain, studied by several authors through the use of different techniques (Mullins et al., 1999; Neumaiera et al., 2001; Shirayama et al., 2001; Bonaventure et al., 2004), demonstrated predominant expression within the hypothalamus, thalamus, hippocampus, cortex, amygdala, and striatum. In line with this ample expression, 5-HT7-R has been linked to several functional roles, such as thermoregulation, learning and memory, hippocampal signaling, circadian rhythm, and sleep (Duncan et al., 1999; Hagan et al., 2000; Ehlen et al., 2001). The receptor is also involved in cognitive and emotional processes, including anxiety and depression (Hedlund and Sutcliffe, 2004; Meneses, 2004; Wesolowska et al., 2006; Mnie-Filali et al., 2011), and it has been recently proposed for a role in neurogenesis, synaptogenesis and dendritic-spine formation, especially during development (Kobe et al., 2012; Rojas et al., 2014).

This complex spectrum of functions attracted a great interest for the development of chemicals able to modulate the activity of this receptor in a targeted fashion. In recent years, different selective 5-HT7-R antagonist and agonist compounds have been developed. Among the latter, LP-211 is a novel agonist whose *in-vitro* pharmacological properties make it suitable for possible psychoactive effects (Leopoldo et al., 2008, 2011; Hedlund et al., 2010). Preclinical evidence in mice demonstrates consistent acute/sub-chronic effects onto exploratory

motivation, anxiety-related profiles, and spontaneous circadian rhythm (Adriani et al., 2012; Romano et al., 2014). Moreover, given the putative role of 5-HT7-R in neural plasticity during development, we recently postulated that receptor stimulation may result in neuro-plastic changes leading to a persistent alteration on forebrain circuits. Such developmental effects of LP-211, originated by adolescent subchronic exposure, have been recently demonstrated in rats using either a behavioral approach (Ruocco et al., 2014a,b) or several different *in-vivo* magnetic resonance (MR) techniques (Canese et al., 2014).

The pharmacological MRI (phMRI), an application of functional MRI (fMRI), has started to represent a robust approach for the direct observation of drug action within the central nervous system (Gozzi et al., 2008; Kocsis et al., 2013), and—in the last years—it has become increasingly popular due to its non-invasiveness and relative low cost. The signal measured by either fMRI or phMRI is determined by local changes in the ratio of oxygenated to deoxygenated hemoglobin, and it is known as Blood Oxygenation Level Dependent (BOLD) signal. In response to neuronal activity, the locally elevated oxygen consumption leads to a feedback increase of blood flow, resulting in a rise of blood-oxygenation levels. In phMRI, changes in the BOLD signal are observed in response to a pharmacological stimulus. A previous study (Canese et al., 2011) revealed different phMRI responses to selective or non-selective blockade of serotonergic pathways, suggesting that the use of selective compounds, and noteworthy agonists such as LP-211, could give a deeper insight into the serotonin system.

In this work, we investigated the differential response to acute LP-211 administration in rats, as a function of adolescent subchronic exposure to the same drug. The use of phMRI allowed us a deeper view into persistent, long-term changes triggered by developmental 5-HT7-R stimulation, by investigating the carry-over sensitization of acute, hemodynamic effects induced by LP-211. Only those brain areas, displaying a differential drug-evoked phMRI signal as a function of pre-treatment, were then selected for *ex vivo* histological and immuno-histochemical analyses, in order to evaluate underlying neuro-anatomical and neuro-physiological changes.

MATERIALS AND METHODS

All experimental procedures were approved by the Institutional Animal Survey Board on behalf of the Italian Ministry of Health (formal license to G.L.). Procedures were in close agreement with the European Communities Council Directive (86/609/EEC) and Italian law. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in-vivo* techniques, if available.

SUBJECTS

Twenty male Wistar rats from our colony were housed in pairs inside polycarbonate cages (42.5 × 26.6 × 18.5 cm) with sawdust bedding, in an air-conditioned room (21 ± 1°C, relative humidity 60 ± 10%), on a 12-h reversed light-dark cycle (lights off at 7.00 a.m.). Food (Altromin-R, A. Rieper S.p.A., Vandoies, Italy) and tap water were provided *ad libitum*.

PHARMACOLOGICAL TREATMENT

Rats were randomly assigned to receive a sub-chronic intraperitoneal (i.p.) administration of LP-211 (LP, 0.250 mg/kg/day, $n = 10$), or vehicle (VEH, 1% DMSO in saline, $n = 10$) for 5 days during the adolescent phase (43–47 post natal days, PND). The drug dosage was chosen according to previous data obtained in mice and rats (Adriani et al., 2012; Canese et al., 2014; Romano et al., 2014; Ruocco et al., 2014a,b).

At adulthood (63–75 PND; weight range: 340–430 g), rats underwent phMRI, with an acute challenge of either LP-211 (10 mg/kg i.p.), or vehicle. Our aim was to measure acute response to LP-211 challenge, modulated by the different pre-treatments. Animals were therefore subdivided into three final experimental groups, according to the substance administrated in the adolescent phase (subchronic pre-treatment) and during phMRI analyses (acute administration): 1. the adolescent subchronic VEH + acute VEH (VEH+VEH) and the adolescent subchronic LP-211 + acute VEH (LP+VEH) were collapsed, generating a single "vehicle-challenge" group (VEH), since no differences between these two groups were observed in preliminary phMRI data analyses; 2. adolescent subchronic VEH + acute LP-211 (VEH+LP: acute challenge in drug-naive rats); 3. adolescent subchronic LP-211 + acute LP-211 (LP+LP: sensitized response to the challenge).

PREPARATION OF ANIMALS FOR phMRI

Anesthesia was induced with isoflurane (Esteve veterinaria, Spain, 5% for induction and 2.5% during the set-up) in O_2 and then rats were intubated and mechanically ventilated [90 bpm, volume depends on animal weight following the formula: strokes volume (ml) = animal weight (g) × 0.0062]. Artificial ventilation allows triggering all functional acquisitions at the same instant of breath cycle, thus minimizing motion artifacts and preventing drug-induced alterations in the respiratory rate when the LP-211 challenge is injected.

Rats were fixed on the cradle using a stereotaxic head frame to reduce head movements. A cannula was inserted subcutaneously to administrate a bolus of medetomidine (Domitor, Pfizer Germany, 0.05 mg/kg in 0.5 ml) and then connected to the infusion line for continuous medetomidine administration (0.1 mg/kg/h in 1 ml/h). The continuous infusion of the anesthetic started 15 min after bolus; at the same time, isoflurane was reduced to 0.6%. A second cannula was inserted into the peritoneum and connected to an infusion line for remote drug administration during the MRI session. An integrated heating system allowed maintaining the animal body temperature at 37.0 ± 0.1°C.

Rats were monitored during MR scanning using a MRI-compatible pulse oximeter (MouseOx, Starr Life Sciences Corp) that provides on-line measures of heart rate, oxygen saturation, and pulse distension (a surrogate parameter for blood pressure; its measured values depend on the intensity of the pulse-oximeter signal, therefore we normalized its actual value to a percentage of maximum in the time course of each animal). After the scan, medetomidine was antagonized using the antidote, atipamezole (Antisedan, Pfizer Germany, double dose with respect to medetomidine in 0.5 ml s.c.) that ensures a fast awakening and recovery

of all animals. A wash out period of at least 1 month was left before sacrifice.

phMRI PROTOCOL AND ACQUISITION

Experiments were performed on a VARIAN/Agilent Inova MRI/MRS system operating at 4.7 T equipped with an actively shielded gradient system (max 120 mT/m, 11 cm bore size). A volume coil of 6-cm diameter was used for transmission in combination with an electronically decupled receiver-only surface coil (Rapid Biomedical, Rimpar, Germany). The shape of this receiver coil (3 cm long, 3 cm wide, and 1 cm high) was designed to optimally fit the dorsal surface of the rats' heads centered over the forebrain regions.

Gradient echo scout images were used to detect position of each animal's head inside the magnet. Then, fast spin-echo sagittal anatomical images (TR/TEeff = 3000/15 ms, 15 consecutive slices of 1 mm thickness, FOV = $40 \times 40\,mm^2$, matrix of 256×256, 4 averages, voxel resolution = $0.31 \times 0.31 \times 1$ mm) were used to accurately position axial images for the pharmacological study. In order to have a reproducible positioning of acquired slices, we considered the forceps minor of the corpus callosum as reference (Canese et al., 2009) as shown in **Figure 1**.

Echo planar breath-triggered images were acquired ($TR \approx$ 4000 ms, $TE = 23$ ms, matrix 64×64, FOV $25 \times 25\,mm^2$, slices: 6, thickness 1 mm, 1 average). After consecutive image collection for about 12 min (20 baseline images), rats received LP-211 (10 mg/kg i.p.) or vehicle (1 ml/200 g body weight). Images were then collected for further 24 min (40 post-challenge images).

phMRI DATA AND STATISTICAL ANALYSES

Data were analyzed by a home-made program (developed in Matlab, Mathworks Inc.) as previously described (Canese et al., 2009, 2011). During the pre-processing, images were realigned in order to reduce the artifacts due to animal movements during data acquisition (Canese et al., 2009). Once the images were realigned, they were then restored in order to increase the signal-to-noise ratio (SNR). In order to efficiently smooth the rough data, we implemented a procedure based on a moving average data interpolation. Then, two-step statistical approaches were conducted in parallel (Canese et al., 2011) on the acquired datasets.

First step

We constructed a template for each group: after selecting the dataset of one subject as a reference for the group, the datasets of the other subjects were transformed by means of a poly-nomial transformation (Goshtasby, 1988). Once the image sequences had been co-registered to the reference images, the software averaged the whole data acquired from all individual datasets of each group in order to obtain one template per group. Within each template, significant temporal alterations in BOLD signal profiles were assessed pixel-by-pixel, through a random effect analysis comparing a post challenge time window (five-point wide window centered on the signal maximum) to the mean baseline signal, with a two tailed Student t-test ($p < 0.065$, Bonferroni corrected).

Second step

We extracted BOLD signal intensity timecourses, i.e., one curve for each individual animal, from several brain areas. These regions-of-interests (ROIs) were selected (manually, on the template images, **Figure 2**) based on those areas showing apparent activation in LP+LP but not VEH+LP templates. We therefore selected: hippocampus (Hip) in slice 2; septum, dorsal striatum (Str) and frontal cortex in slice 4; medial prefrontal cortex, dorsolateral prefrontal cortex and nucleus accumbens (NAcc) in slice 6, according to the activation maps of group templates.

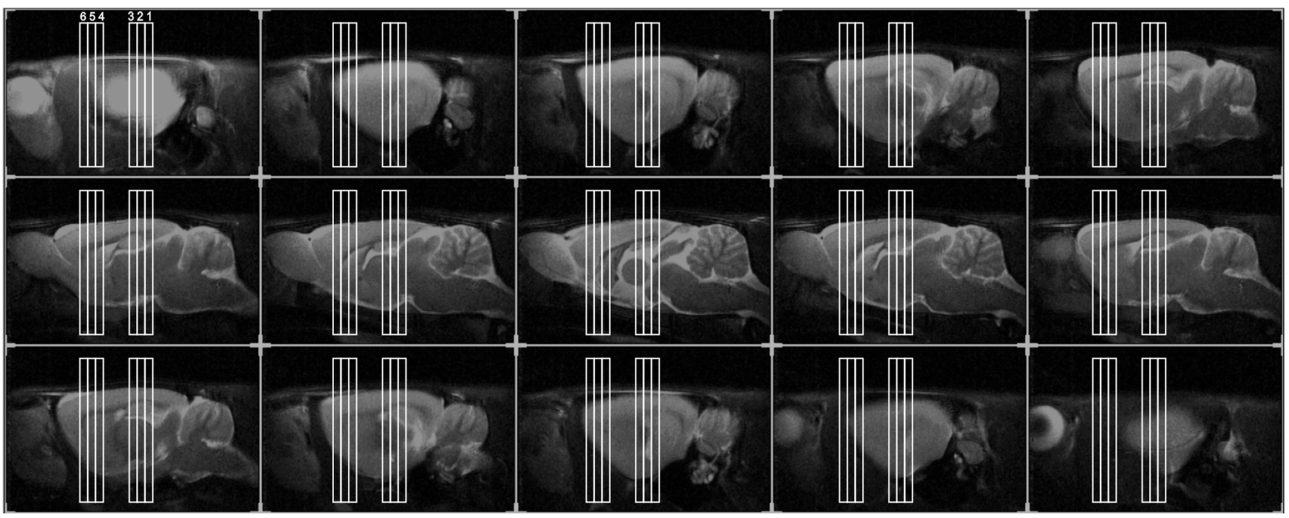

FIGURE 1 | Positioning of the six slices for phMRI, overlaid on the anatomical sagittal fast spin-echo images taken from an individual representative rat. We acquired six slices in total: three consecutive slices (thickness 1 mm), centered on the hippocampus (Hip, slice 1, 2, 3), as well, after a gap of 3 mm, other three slices. These were centered, respectively, on: dorsal striatum (dStr, slice 4), on the fibers between the prefrontal cortex and striata (slice 5), on prefrontal cortex (PFC, slice 6).

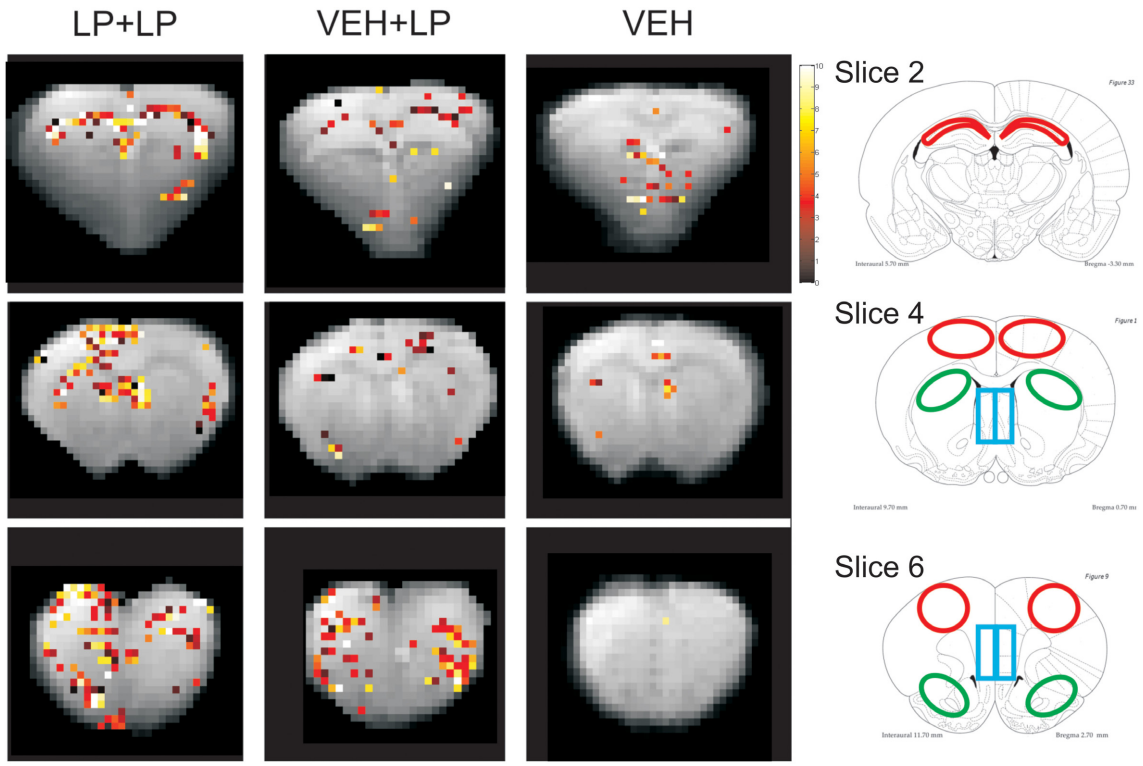

FIGURE 2 | Right panels: localization of ROIs. Left panels: BOLD activation maps (the Student-*t*-values, in color scale), overlaid on anatomical images (in gray scale). Both are taken from the three brain templates, originating from rats receiving: vehicle challenge (VEH), LP-211 acutely only (in drug-naive subjects) i.e., following adolescent vehicle exposure (VEH+LP), LP-211 also in acute but following adolescent subchronic pre-treatment (LP+LP). Only pixels with significant values ($p < 0.065$ for slice on Hip and $p < 0.05$ for the others) are illustrated. ROIs were taken with the help of the Atlas (Paxinos and Watson, 1998) from: the CA1-CA2 fields of Hip (red regions at −3.30 from bregma, slice 2); from the dSTR, septum and frontal cortex (see green, blue, red ROIs, respectively, at +0.7 from bregma, slice 4); from the nucleus accumbens, medial prefrontal cortex, dorsal prefrontal cortex (see green, blue, red, ROIs, respectively, at +2.7 from bregma, slice 6).

In the timecourses for the extracted BOLD signal curves, each temporal point was obtained by averaging the BOLD signal intensity values across all chosen-ROI pixels at that time point. In such BOLD timecourses, each time point was expressed as the percentage change of the signal intensity from the first time point. These BOLD timecourses underwent normalization of baseline and detrending. In particular, the baseline mean values (estimated as an average of the first 20 time points) were subtracted from each BOLD timecourse. This, in order to decrease inter-individual variability due to random (instrumental and biological) fluctuations in the first timepoint. Then, for each individual rat, a linear regression fit was applied to the first 20 and last 15 time points, in order to estimate the temporal drift of the curve. This drift line was then subtracted from the entire BOLD timecourse (Canese et al., 2009, 2011).

Like for group templates, animals receiving acute vehicle challenge (LP+VEH and VEH+VEH) where merged in a single group (VEH): this, since preliminary analyses yielded no differences between these two groups, as expected. Split-plot ANOVA was thus performed, with group (three levels: LP+LP vs VEH+LP vs VEH) as between subjects factor, time (40 points after injection, 24 min) and side (right vs left hemispheres except for septum and medial prefrontal cortex) as within subject factors. Analyses were performed on the post-challenge curves, in search for drug-induced effects. Tukey's Honestly Significant Difference (HSD) test was used for multiple *post-hoc* comparisons (Abdi and Williams, 2010).

TISSUE SAMPLING AND PROCESSING

At sacrifice, brain hemispheres were fixed in 4% buffered formaldehyde and blind coded brain slices were obtained from four standard coronal sections (2-mm thick). The slices were washed in running water, dehydrated in graded alcohols, cleared in xylene and finally embedded in paraffin wax. 5-μm-thick sections were cut on microtome, and collected onto glass slides for histological and immuno-histochemical analyses.

Histology and immuno-histochemistry

For histological analyses, the slides were deparaffinized, rehydrated, and stained with haematoxylin/eosin or with luxol fast blue/eosin for the precise identification of the brain structures. For immuno-histochemistry, sections were collected on Super Frost Plus slides and immunostained according to the ABC method for brightfield examination.

Briefly, sections were deparaffinized, rehydrated and subjected to antigen retrieval by microwaving in citrate buffer. The slides

were then treated with 3% hydrogen peroxide in methanol to block endogenous peroxidases activity, rinsed in PBS, blocked for 1 h in PBS containing 3% normal goat serum and then incubated overnight at 4°C with one of the following antibodies: 5-HT7-R (cod. NBP1-46598, Novus Biologicals, rabbit, 1:1000 dilution); post synaptic marker PSD95 (cod. NBP1-47642, Novus Biologicals, mouse, 1:500 dilution); neuro-filament NF-L (DA2) (cod. NB 300-132, Novus Biologicals, mouse, 1:250 dilution); GFAP for astrocytes (cod. M0761, DAKO, mouse, 1:100 dilution).

Subsequent antibody detection involved incubation with the appropriate biotinylated secondary antibody for 1 h (1:200 dilution, Vector Laboratories, Italy) at room temperature, followed by incubation with the avidin-biotin-peroxidase complex (Vectastain ABC-Elite kit, Vector Laboratories, Italy) according to the manufacturer's instructions. The samples were stained with 3′-3′diaminobenzidine (DAB, Sigma, Italy) as chromogen to visualize the reaction product and then lightly counterstained with haematoxylin.

For immuno-fluorescence, sections were processed as above but the antibody detection was performed through 1 h of incubation with Alexa Fluor 488 secondary antibody (1:300 dilution, Invitrogen, Italy). In order to obtain comparable data and a more consistent staining, each antibody was tested on both groups in the same immuno-histochemical run.

For the count of 5-HT7-R and GFAP positive cells and for densitometric measures, anatomical landmarks were used as optical reference so that equal areas at the rostrocaudal levels were analyzed for each animal. Three non-consecutive serial sections from each brain were photographed under 20× magnification, with constant light conditions, and the labeled cells were counted manually in selected brain areas (i.e., nucleus accumbens and septum). Staining intensities were quantified on immunofluorescent slides using ImageJ software (NIH, http://rsb.info.nih.gov/ij). 5-HT7-R intensities were calculated using the mean intensity value of the 5-HT7-R positive cells. For that, RGB images were splitted, the green channel was selected and 8-bit converted. Images were then thresholded and ROIs selected for quantification. Optical densities of the ROIs were recorded and expressed as values on a gray scale, ranging from 0 (black) to 255 (white). For statistical analysis, student *t*-test was used.

RESULTS

Heart rate and pulse distension, measured pre and post challenge during the phMRI study, are summarized in **Table 1**. Typical time courses are shown in **Supplementary Figure 1**. No statistical differences were found in the data between the LP-211 challenge and vehicle controls [ANOVA with repeated measurements, $F_{(1,\ 8)} = 2.71$, n.s., $F_{(1,\ 8)} = 0.03$, n.s., respectively], confirming that LP-211 challenge did not alter the depth of anesthesia in rats. Oxygen saturation is not relevant in these experiments because animals were mechanically ventilated with oxygen at 99% (instead of air) during MR measurements. Therefore, this parameter was constant during the whole experiment.

Table 1 | Physiological parameters in rats during Ph-MRI measurements, before (Pre) or after (Post) the challenge administration.

	Heart rate (bpm)		Pulse distension (%)	
Experimental group	**Pre**	**Post**	**Pre**	**Post**
(VEH or LP)+LP	234.36 ± 36.20	218.20 ± 32.61	89 ± 4%	73 ± 9%
VEH	226.70 ± 23.53	219.74 ± 20.58	84 ± 2%	66 ± 10%

A significant reduction in pulse distension between pre and post challenge is present in all groups [time, $F_{(1,\ 8)} = 41.45$, $p < 0.001$]. This reduction cannot be attributed to LP-211 challenge or pre-treatment since it is present also in vehicle controls, and it is probably due to the prolonged anesthesia or to cardiovascular readjustment to the volume of injection. Finally, time courses of physiological parameters measured during MR scan pre and post challenge were compared to time courses of BOLD curves and we found they were temporally uncoupled.

Our findings are not in conflict with the increased wakefullness in rats due to LP-211, as reported in literature (Romano et al., 2014). In fact, in our case, rats were anesthetized with a double anesthetic (isoflurane and medetomidine) that likely overcomes the behavioral effect of LP-211 already reported.

TEMPLATES' ACTIVATION MAPS, BOLD TIMECOURSES

No consistent activation was found in the VEH template. As for drug challenged groups, activation maps showed BOLD signal hyper-intensity within several regions, with different extension and intensity depending on the adolescent, subchronic pre-treatment. Areas of a positive BOLD effect (i.e., regions whose pixels exceeded the threshold of significance) were detected, within the LP+LP but not the VEH+LP group, for the hippocampus (CA1-CA2 fields, slice 2), septum, frontal cortex, and part of dorsal striatum (slice 4), as well as for medial prefrontal cortex, dorso-lateral prefrontal cortex and the nucleus accumbens (slice 6). The orbital prefrontal cortex was the only region showing clear-cut activation in templates for both VEH+LP and LP+LP groups.

Therefore, as shown in **Figure 2**, the VEH+LP group showed a differential response to the challenge with respect to the LP+LP group. In particular, for drug-naive animals receiving LP-211 for the first time as an acute challenge (i.e., VEH+LP template), non-localized and/or widespread effects were observed, with just scattered pixels or reduced extension of activation, in hippocampus and at the level of the nucleus accumbens. In this template, the septum, frontal cortex and dorsal striatum, as well as dorso-lateral and medial prefrontal cortex, did not show any significant response; therefore, for the latter areas, the VEH+LP and VEH templates were undistinguishable, suggesting no effect at all of an acute LP-211 challenge in drug-naive rats.

Localization of the seven regions, for time-courses extraction and analysis, is shown in **Figure 2** right panel. Analyses of the BOLD timecourses, individually extracted from subjects, confirmed a differential activation for just three out of the seven chosen areas (see **Figure 3**): for hippocampus, nucleus accumbens, and septum. Data showed that the BOLD effects differed between LP+LP and VEH+LP experimental groups. Significant

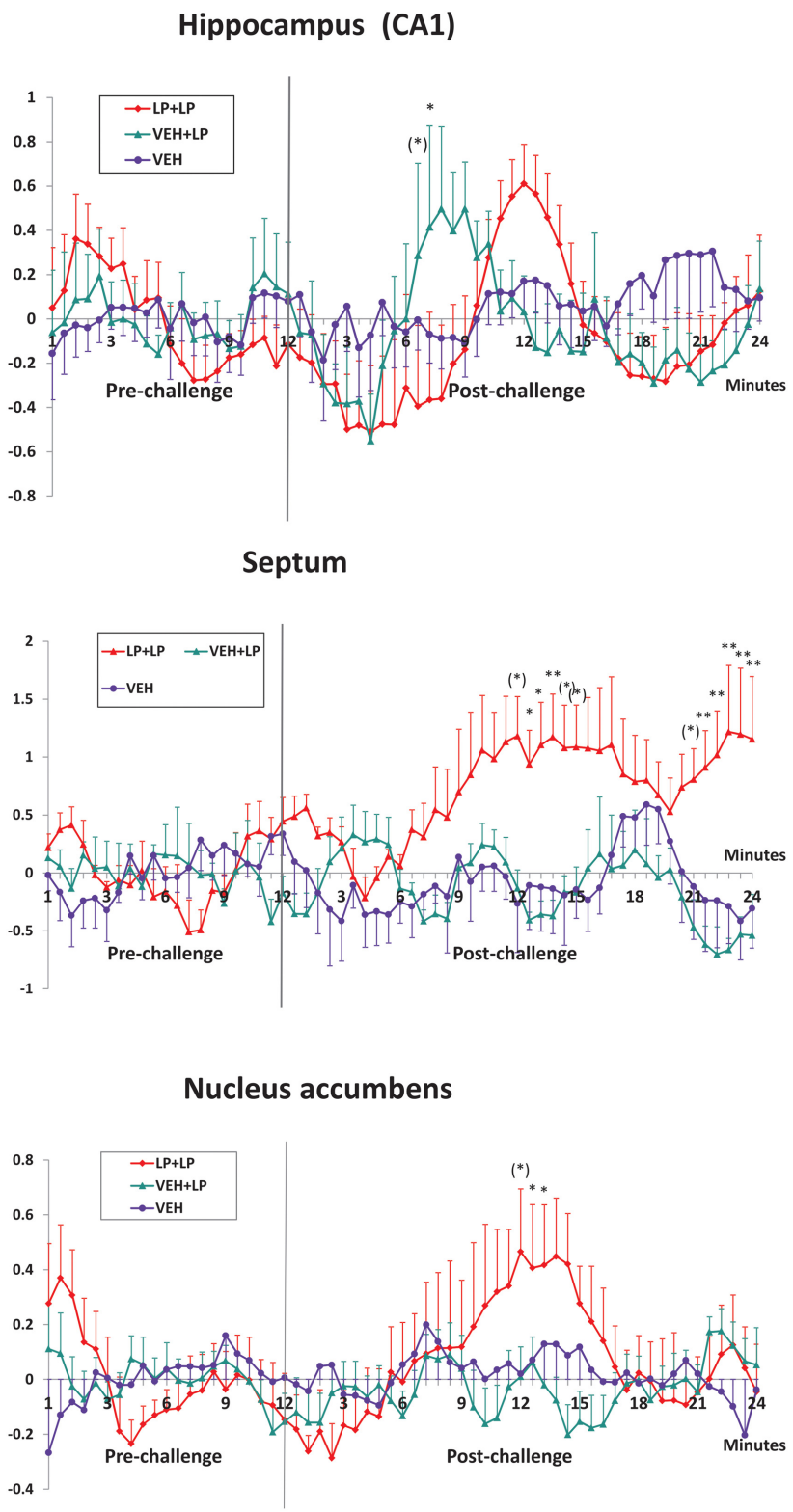

FIGURE 3 | Mean ± s.e.m. change (%) in BOLD signal timecourses, in the CA1-CA2 fields of Hip, septum and nucleus accumbens (ROIs shown in Figure 2), for rats receiving vehicle challenge (VEH), LP-211 acutely only (in drug-naive subjects) i.e., following adolescent vehicle exposure (VEH+LP), LP-211 also in acute but following adolescent subchronic pre-treatment (LP+LP). Time after the challenge is expressed over ten 3-min intervals, obtained by averaging the originally acquired data in ten 5-point-wide time-windows. Significant *post-hoc* Tukey comparison (LP+LP vs VEH+LP groups) are indicated as follows:
(*)$0.05 < p < 0.1$;* $p < 0.05$;* $p < 0.01$.

interactions time × treatment were indeed found only in septum [$F_{(80,\ 360)} = 1.43$, $p = 0.015$], in hippocampus [$F_{(80,\ 360)} = 1.70$, $p = 0.006$], and in nucleus accumbens [$F_{(80,\ 280)} = 1.55$, $p = 0.005$]. For both septum and nucleus accumbens, the *post-hocs* confirmed that a BOLD effect was evident in LP+LP and absent in VEH+LP curves. For the hippocampus, an activation signal was present in both the LP+LP and VEH+LP groups (though with different intensities and temporal outline, see **Figure 3**). This, together with the scattered points already mentioned by VEH+LP template inspection, suggested that this area was indeed activated in drug-naive rats, after a single acute LP-211 administration.

For medial and dorso-lateral prefrontal cortex, frontal cortex, as well as dorsal striatum (all of which appeared to be activated on LP+LP template but clearly devoid of activated pixels on VEH+LP template), ANOVAs did not confirm the profile (data not shown): as such, the pixels activated on template may well be a false positive. The ANOVA on timecourses was not necessary for orbital prefrontal cortex, as this area was clearly activated in both LP+LP and VEH+LP groups.

EX VIVO HISTOLOGY AND IMMUNO-HISTOCHEMISTRY

We performed histological examinations of luxol fast blue and haematoxylin-eosin stained slides obtained throughout the whole brain. This morphological analysis showed no relevant alteration in LP+VEH vs VEH+VEH animals. In-depth immunohistochemical analyses, for the evaluation of neuro-anatomical and neuro-physiological changes, were then conducted in two selected brain areas. We chose the nucleus accumbens and the septum, the only two regions with positive phMRI activations for the LP+LP group only, confirmed both in template maps and in BOLD timecourse analyses (depicted in **Figures 2**, **3**). These areas could possibly have developed stable neuro-anatomical or neuro-physiological changes, induced by subchronic treatment with LP-211 during adolescence.

The *ex vivo* investigation for prefrontal and frontal cortex regions as well as dorsal striatum was not performed, because these areas resulted not to significantly differ, as a function of pre-treatment, in the ANOVA analyses. Conversely, for the orbital prefrontal cortex and the hippocampus, activation signal was present, after acute LP-211 administration, in both the LP+LP

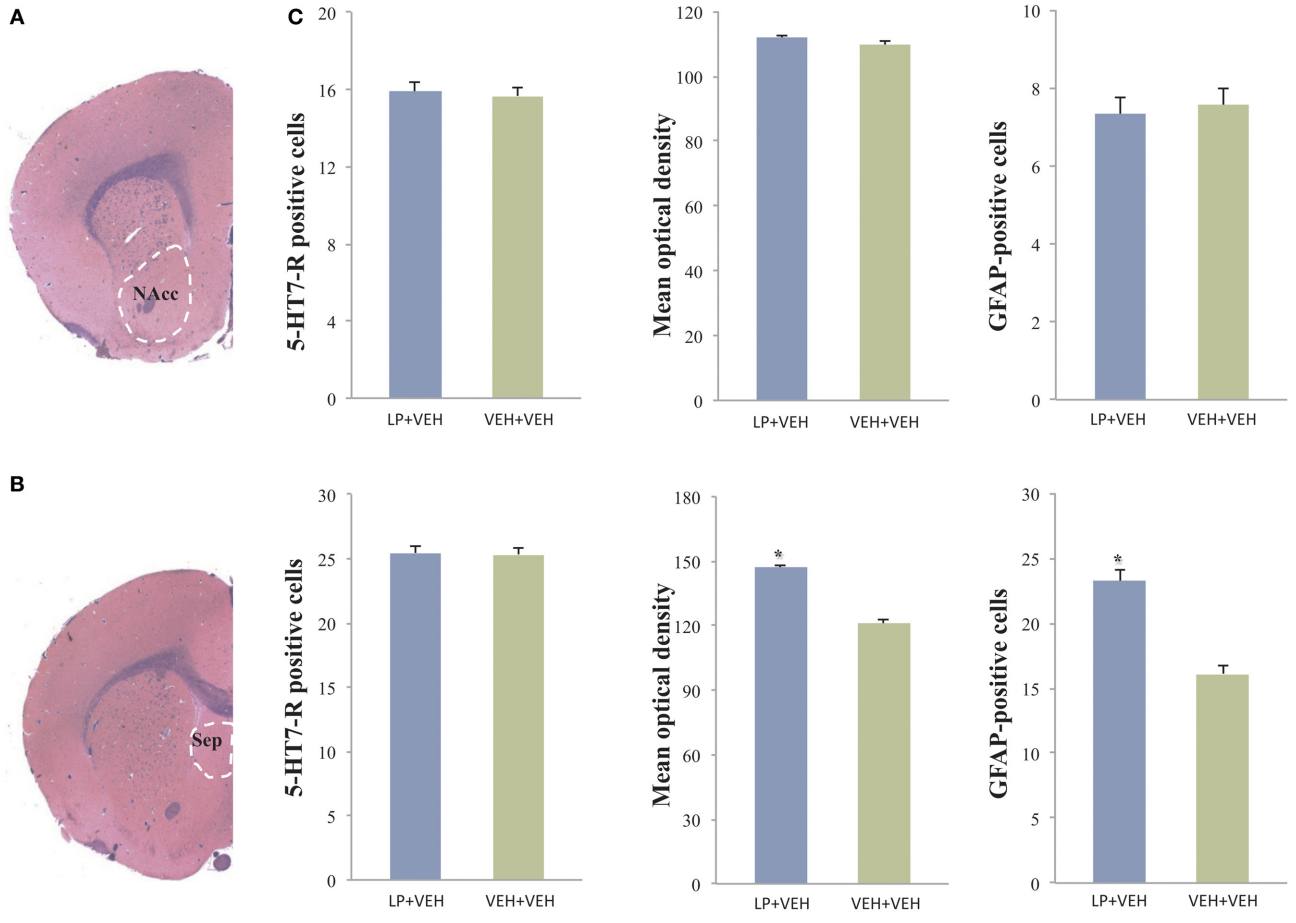

FIGURE 4 | Analysis of 5-HT7-R and GFAP immunoreactivity in the nucleus accumbens (NAcc) and in the septum: statistically significant differences were observed only in the septum. Here, animals subchronically pre-treated during adolescence with LP-211 drug showed an increased 5HT7-R optical density and an increased number of GFAP-positive cells with respect to VEH-exposed control group **(C)**. The values are given as mean ± *SE*; asterisks denote a statistically significant difference ($p < 0.05$). No differences for 5HT7-R were observed by cell count in both areas. **(A,B)** are representative photomicrographs at the level of the NAcc and septum in Luxol fast blue-stained slides.

and VEH+LP groups; therefore, an *ex vivo* analysis in these two regions was out of the purposes of present study.

Regarding the 5-HT7-R expression, counting of immunoreactive cells in the septum revealed no differences in the total number of 5-HT7-R expressing cells between the two groups. Conversely, the analysis of the 5-HT7-R optical density revealed a significant increase in immunoreactivity for the group subjected to adolescent pre-treatment, compared to adolescent VEH-exposed controls (**Figure 4**). These results indicate increased levels of receptors expressed by septal cells, as a long-term consequence of adolescent LP-211 treatment. Besides the 5-HT7-R changes, we found an increase in GFAP immunoreactivity (**Figure 5**), suggesting persistent rearrangement with an increased response of astroglial cells in adolescent-LP-211 pre-treated animals. No differences were observed with the post-synaptic and with the neuro-filament markers.

In the nucleus accumbens, neither the cell counts nor optical density analyses of 5-HT7-R positive cells revealed any difference between adolescent drug pre-treated animals and VEH-exposed controls (see **Figure 4**); likewise, no differences were observed with the other markers. Such results suggest that the subchronic pre-treatment with LP-211 during adolescence does not produce an immuno-histochemically detectable effect on this brain area, at least regarding the selected markers.

DISCUSSION

The present work was aimed to study the long-term sensitizing effects of the adolescent, subchronic administration of LP-211, a novel 5-HT7-R agonist. To do this, a two-level approach was adopted: first, adult rats underwent phMRI analyses (with an LP-211 challenge) following a previous adolescent exposure, to identify LP-211 sensitized areas which were not acutely responsive in drug-naive rats; once identified, these (two) areas were studied *ex vivo*, at the histological and immuno-histochemical level, to detect the presence of stable neuro-anatomical and biochemical changes.

General anesthesia is usually induced in rodents during phMRI studies in order to reduce motion artifacts, to minimize the stress produced by prolonged restraint and MRI gradient noise, and to facilitate animal handling (Lukasik and Gillies, 2003; Sicard et al., 2003; Steward et al., 2005). Unfortunately, general anesthesia may perturb cerebral blood flow and metabolism, as well as electro-physiological, cardio respiratory, and other physiological parameters; finally, it can suppress neuronal activity reducing the BOLD response (Gozzi et al., 2008). Recent studies reveal that BOLD response to the same challenge may change from negative to positive, or differ in amplitude, with different anesthetic regimes (Sommers et al., 2009; Hodkinson et al., 2012; Liu et al., 2012). Also, the pharmacological challenge in itself can produce cardio respiratory physiological responses that may alter hemodynamic measurements in brain. The particular sensitivity of phMRI to natural and anesthesia-induced fluctuations in vital parameters is a well-documented issue, affecting the quality and interpretation of phMRI results. For such reasons, in the last years several studies have tried experimental procedures to achieve the most reliable and reproducible phMRI acquisition protocol (Steward et al., 2005; Weber et al., 2006; Ferrari et al., 2012).

For these reasons, the present work exploited a protocol for sedation and anesthesia consisting of two anesthetic compounds (isoflurane and medetomidine), under continuous supply, associated with intubation, and mechanical ventilation, to assure stability in physiological parameters: such a protocol is suitable for prolonged investigations (Weber et al., 2006; Fukuda et al., 2013). This protocol also assures a fast recovery of the animals and therefore it is suitable for longitudinal studies as well as for further behavioral testing (Canese et al., 2014). Once optimized, this setting (anesthetic regimen combined to the respiratory trigger) allowed to acquire phMRI scans of the desired resolution, with signal alterations as low as 0.5% and strongly reduced motion artifacts.

Moreover, physiological parameters (heart rate and pulse distention) measured during MR scan, pre and post challenge, did not show differences due to LP-211 administration. We can exclude any bias due to cardiovascular rearrangements or systemic alterations, and therefore attributed to LP-211 any effect observed in BOLD signal variations.

In this way, we found robust and significant results by using and comparing data obtained with two different and parallel statistical approaches (Canese et al., 2011). By using this combined approach, we were able to identify significant alterations in several forebrain areas. The orbital prefrontal cortex and hippocampus were the only regions showing a clear-cut activation due to drug challenge, with no differences between adolescent-LP-211 pre-treated (LP+LP) and adolescent-VEH exposed (VEH+LP) groups; therefore, these were not further investigated *ex vivo*. Conversely, in the absence of acute response to LP-211 (10 mg/kg i.p.) within drug-naive subjects, two areas showed a sensitized response to the same drug due to previous developmental exposure. Indeed, in the septum as well as in the nucleus accumbens, BOLD response was evident within the LP+LP group but not in the VEH+LP one. A somewhat similar profile seemed to emerge for frontal and prefrontal cortex as well as (part of) dorsal striatum, at least in template activation maps, but this was not confirmed by ANOVA analyses on BOLD timecourses.

The suggestion of a persistent modification in neurocircuitry, supported by recent data (Canese et al., 2014), was further examined with immuno-histochemistry. In the absence of detectable changes for the nucleus accumbens, the septum revealed the most interesting differences between adolescent-LP-211 pre-treated vs. adolescent-VEH exposed rats after *ex vivo* immuno-histochemical examination. In particular, sub-chronic administration of LP-211 during adolescent phase exerted a direct and long-lasting effect in the septal area, with a specific increase of 5-HT7-R immunoreactivity coupled with an increased astrocytes response (as observed by GFAP immunoreactivity).

These two aspects may well be related. The observed increase in astrocytes response within the septum could represent a specific, yet secondary response to an increased 5-HT7-R immunoreactivity in local neurons, produced by the LP-211 adolescent pre-treatment. Together with this, it is possible that the increased 5-HT7-R immunoreactivity, observed in the septum, derives, at least in part, from an increased receptor expression in activated astrocytes. Consistently, 5-HT7 receptors have been classically

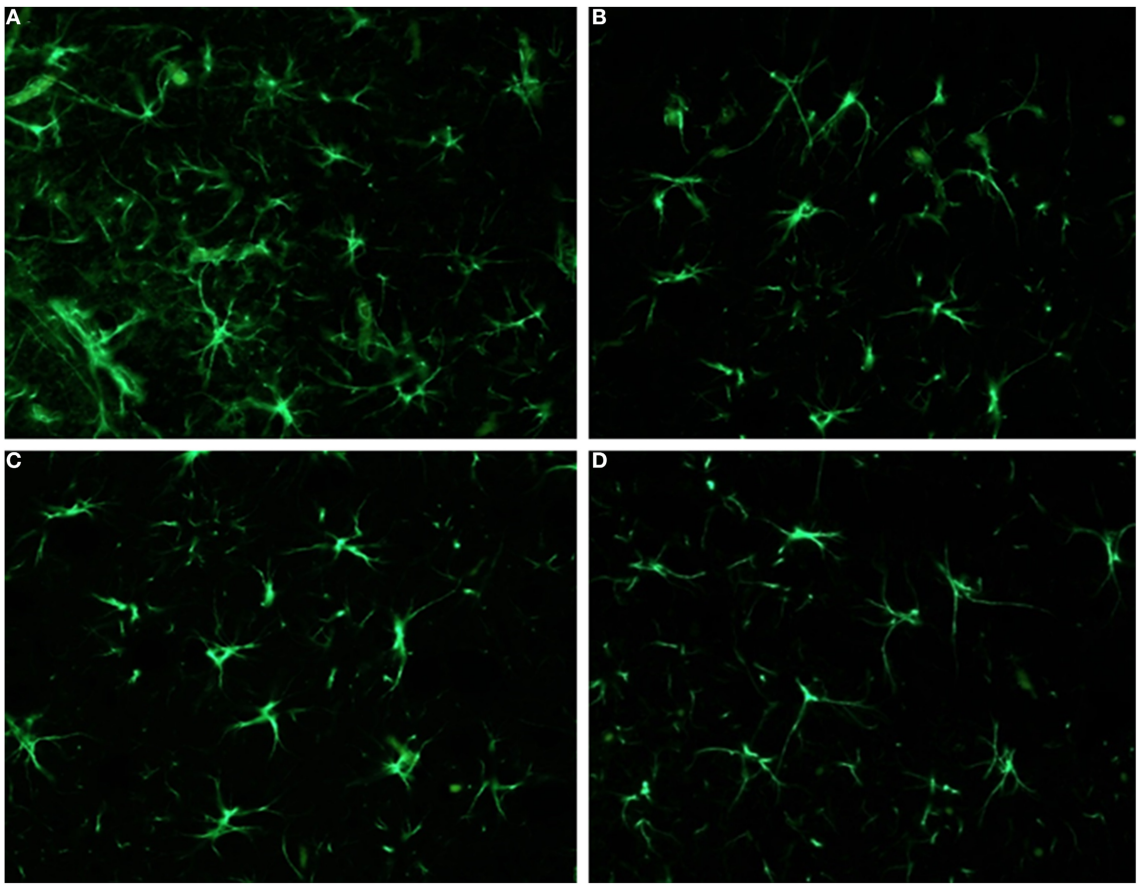

FIGURE 5 | Analysis of GFAP immunoreactivity in the nucleus accumbens (NAcc) and in the septum: significant differences were observed only in the septum. Here, animals subcronically pre-treated during adolescence with LP-211 drug **(A)** revealed an increased GFAP immunoreactivity, with respect to the VEH-exposed control group **(B)**. No differences were conversely observed in the nucleus accumbens **(C,D)**. Original magnifications 40×.

reported in glial processes within the supra-chiasmatic nucleus (Glass and Chen, 1999; Belenky and Pickard, 2001), in astrocytes from the frontal cortex (Shimizu et al., 1996), and in primary astrocyte cultures (Hirst et al., 1997).

The idea of astrocytes as primary players in the pathophysiology of the CNS is not new: astrocytes are key elements that participate in all essential CNS functions. They represent a wide and heterogeneous population of cells whose activity is finely regulated on the basis of specific functions and responses of their relevant neuronal population (Fellin, 2009). In recent years, astrocytes are emerging as relevant and active elements in brain physiology, where they act by exchanging information with the synaptic neuronal elements, responding to synaptic activity and regulating synaptic transmission. The term "tripartite synapse" is now used to represent the bi-directional communication between astrocytes and neurons at the synapse's interface (Araque et al., 1999). In line with this, a major role of astrocytes is the uptake of neuro-transmitters, mainly glutamate and GABA, therefore contributing to modulate local network transmission. Variations in neuro-transmitter levels (mainly glutamate) were already observed, after adolescent subchronic LP-211 administration, in previous work by our group: through amino-acid detection *ex vivo*, within prefrontal and striatal areas (Ruocco et al., 2014a,b) and through MR spectroscopy, within the hippocampus (Canese et al., 2014). We could speculate that similar variations might also occur in the septum, and this would represent a further stimulus for an increased astrocytes immunoreactivity. We did not observe any variation in neuro-filament and synaptic markers, supporting the conclusion of a predominantly glial modification.

Surprisingly, we have not found immuno-histochemical variations in the nucelus accumbens, the other area where sensitized BOLD responses to LP-211 were observed with our phMRI analyses. Although undetectable in our hands, we cannot exclude that adolescent LP-211 administration resulted in variations of the same markers, which were below the detectable level with immuno-histochemistry but would become evident if tested with other techniques. In any case, a set of morphological and functional changes (in the dendritic tree and network connections) were already revealed for the nucleus accumbens in our previous work (Canese et al., 2014). It is therefore possible that, in the absence of changes on the selected markers, persistent modifications could subtend the activation of other proteins or effector patterns downstream of the 5-HT7-R activation, and/or the

mirroring of drug-evoked effects originated from other areas then projecting into the nucleus accumbens, as opposed to the direct effect clearly observed in the septum. Accordingly, we found in our previous work (Canese et al., 2014) that long-term functional modifications, produced by adolescent LP-211, were tapping onto the limbic loop in general and nucleus accumbens in particular. If so, the altered phMRI signal could be interpreted as an indirect effect (of the adolescent LP-211 administration), primarily evoked in another node of the loop (e.g., the hippocampus or orbital prefrontal cortex, actually stimulated). Conversely, while the septum was persistently modified in its anatomy (present data), it did not emerge as part of the functional connectivity alterations induced by adolescent LP-211 in our previous data (Canese et al., 2014).

As a final remark about drug dosage, LP-211 (10 mg/kg) has been used in various *in vivo* studies (Hedlund et al., 2010; Martinez-Garcia et al., 2014; Meneses et al., 2014), and effects possibly mediated by the activation of 5-HT1A or 5-HT2B receptors were not observed (i.e., body temperature, cardiac failure, respectively). Therefore, although the dosage is relatively high, we can confidently assume that LP-211 is still selective to 5-HT7 receptor.

CONCLUSION

In summary, our results suggests that subchronic LP-211 administration during the adolescent phase is able to induce physiological changes in the 5-HT7-R expression and in astrocytes response, within the septum, that can still be observed in adulthood. The increased number of GFAP-positive cells within the septum, found in the present work, is not yet related to the evidence of a rearranged connectivity, recently found within the whole limbic loop (Canese et al., 2014). Therefore, further studies are required to better clarify the nature and the output of such enduring changes.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnbeh.2014.00427/abstract

Supplementary Figure 1 | An example of physiological time courses in a LP-211 challenged rat. After drug administration, there is only a transient increase of pulse distension and a slight and stable increase of heart rate. None of these time courses is coupled with the BOLD one, thus excluding any bias due to systemic effects.

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Selective pharmacological blockade of the 5-HT7 receptor attenuates light and 8-OH-DPAT induced phase shifts of mouse circadian wheel running activity

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Recent reports have illustrated a reciprocal relationship between circadian rhythm disruption and mood disorders. The 5-HT7 receptor may provide a crucial link between the two sides of this equation since the receptor plays a critical role in sleep, depression, and circadian rhythm regulation. To further define the role of the 5-HT7 receptor as a potential pharmacotherapy to correct circadian rhythm disruptions, the current study utilized the selective 5-HT7 antagonist JNJ-18038683 (10 mg/kg) in three different circadian paradigms. While JNJ-18038683 was ineffective at phase shifting the onset of wheel running activity in mice when administered at different circadian time (CT) points across the circadian cycle, pretreatment with JNJ-18038683 blocked non-photic phase advance (CT6) induced by the 5-HT1A/7 receptor agonist 8-OH-DPAT (3 mg/kg). Since light induced phase shifts in mammals are partially mediated via the modulation of the serotonergic system, we determined if JNJ-18038683 altered phase shifts induced by a light pulse at times known to phase delay (CT15) or advance (CT22) wheel running activity in free running mice. Light exposure resulted in a robust shift in the onset of activity in vehicle treated animals at both times tested. Administration of JNJ-18038683 significantly attenuated the light induced phase delay and completely blocked the phase advance. The current study demonstrates that pharmacological blockade of the 5-HT7 receptor by JNJ-18038683 blunts both non-photic and photic phase shifts of circadian wheel running activity in mice. These findings highlight the importance of the 5-HT7 receptor in modulating circadian rhythms. Due to the opposite modulating effects of light resetting between diurnal and nocturnal species, pharmacotherapy targeting the 5-HT7 receptor in conjunction with bright light therapy may prove therapeutically beneficial by correcting the desynchronization of internal rhythms observed in depressed individuals.

Keywords: circadian rhythms, 5-HT7 receptor, phase shift, photic, non-photic, depression, mood, serotonin

INTRODUCTION

Circadian rhythms are governed by a variety of environmental inputs including light, food, social interaction, and pharmacological agents (Lall et al., 2012; Bloch et al., 2013; Patton and Mistlberger, 2013; Pendergast and Yamazaki, 2014). Of these, light is the most powerful of the synchronizing agents (Klein et al., 1991) and exerts its influence on circadian rhythms by first exciting a subset of retinal ganglion cells that subsequently activate neurons within the master circadian clock located in the suprachiasmatic nucleus (SCN) via the retinohypothalamic tract (RHT; Moore and Lenn, 1972; Gooley et al., 2001; Hattar et al., 2002; Panda et al., 2002; Ruby et al., 2002). In addition to photic input, serotoninergic pathways can also exert non-photic influence over the synchronization of circadian rhythms either by direct projections from raphe nucleus onto the SCN or indirect via the intergeniculate leaflet onto the SCN (Moga and Moore, 1997; Pickard and Rea, 1997; Ciarleglio et al., 2011). Working in accordance with each other, the photic and non-photic pathways help ensure proper synchronization of circadian rhythms by responding to a variety of changes in the environment. Recent studies have demonstrated that the misalignment or the inability to adjust to such derivations in the environment give rise to mood disorders in humans (Sprouse, 2004; Grandin et al., 2006; Murray and Harvey, 2010; Salvadore et al., 2010).

Utilizing a "clock in a dish" model, a role for the 5-HT7 receptor in the modulation of the non-photic regulation of circadian rhythms was noted for the first time when the receptor was initially cloned (Lovenberg et al., 1993). This method demonstrated that the phase advance in neuronal activity of the SCN following administration of the 5-HT1A/7 receptor agonist 8-OH-DPAT was attenuated by the 5-HT7/2 receptor antagonist ritanserin, but

not by pindolol, a 5-HT1A/1B receptor antagonist (Lovenberg et al., 1993). This finding was later confirmed with the more selective 5-HT7 receptor antagonist SB-269970 (Sprouse et al., 2004). *In vivo* studies later reported the translational aspects of this cell model by demonstrating that phase advances in wheel running activity induced by 8-OH-DPAT were blocked following the administration of the 5-HT7 receptor antagonist DR-4004 in hamsters and absent in the 5-HT7 receptor knockout (KO) mouse (Ying and Rusak, 1997; Ehlen et al., 2001; Horikawa and Shibata, 2004; Gardani and Biello, 2008; Horikawa et al., 2013). In addition to its effects on the non-photic regulation of circadian rhythms, there is evidence that the 5-HT7 receptor may also influence photic regulation of circadian rhythms either by altering the sensitivity to light or modulating the release of serotonin (Ying and Rusak, 1997; Smith et al., 2001).

In addition to regulating circadian rhythms, the 5-HT7 receptor has been studied extensively for its role in depression. Initial investigation noted that 5-HT7 receptor KO mice exhibited an antidepressant-like phenotype in models of depression such as the tail suspension and forced swim tests (Guscott et al., 2005; Hedlund et al., 2005). Comparable antidepressant-like effects were found in these behavioral tests with the selective 5-HT7 receptor antagonist SB-269970 (Wesołowska et al., 2006; Sarkisyan et al., 2010). While many studies have utilized SB-269970 as a tool compound to investigate a role for the 5-HT7 receptor in various physiologic systems and pathological states, its utility is hampered due to a short half-life and poor drug-like properties (Hagan et al., 2000). Our in-house efforts yielded JNJ-18038683, a selective 5-HT7 receptor antagonist that exhibits better pharmacokinetic properties than SB-269970. Pre-clinical and clinical evaluation of the compound demonstrated that JNJ-18038683 was efficacious in the mouse tail suspension test and also enhanced serotonin transmission, antidepressant-like properties, and REM sleep suppression induced by the selective serotonin reuptake inhibitor (SSRI) citalopram in rats (Bonaventure et al., 2012). The effects of JNJ-18038683 on REM sleep translated from rodents to humans whereas the antidepressant efficacy needed to be further assessed (Bonaventure et al., 2012). Interestingly, systemic administration of the selective 5-HT7 receptor agonist LP-211 significantly increased the time spent awake while the direct infusion of this compound into dorsal raphe nucleus, locus coeruleus, basal forebrain, or laterodorsal tegmental nucleus resulted in decreased duration of REM sleep (Monti et al., 2008, 2014; Monti and Jantos, 2014). Similar REM sleep suppressive effects were observed when another selective 5-HT7 receptor agonist LP-44 was injected directly into the dorsal raphe nucleus (Monti et al., 2008).

Given the association of the 5-HT7 receptor with mood and circadian rhythms, pharmacological manipulation of this receptor may provide a critical insight into the therapeutic link between depression and circadian disruption. Therefore, the current study was designed to examine a role for the 5-HT7 receptor in circadian rhythm regulation by administering the selective 5-HT7 receptor antagonist JNJ-18038683 in both photic and non-photic circadian paradigms. To determine if JNJ-18038683 exerts direct phase resetting properties, a phase response curve was generated by administering the compound to mice at select times throughout the circadian cycle. Second, mice were administered JNJ-18038683 to determine if the compound alters the non-photic phase shift of wheel running activity induced by 8-OH-DPAT. Finally, JNJ-18038683 was administered prior to a light pulse that occurred at times known to delay (circadian time (CT) 15) or advance (CT 22) the onset of wheel running activity (Daan and Pittendrigh, 1976; Takahashi et al., 1984) in mice to determine the effects of acute pharmacological blockade of the 5-HT7 receptor on photic induced phase shifts. Results of these studies demonstrate that the 5-HT7 receptor influences both photic and non-photic aspects of circadian regulation and therefore may provide a therapeutic avenue to alleviate circadian disruptions associated with depression.

METHODS

ANIMALS

Studies conducted for the current investigation were performed in accordance with the policies and regulations of the respective IACUC committees at Northwestern University and Janssen Research and Development, L.L.C. For the experiments outlined for the following studies, male C57Bl/6 J mice (average weight ∼30 grams) were purchased from Jackson Labs (Sacramento, CA) and allowed to acclimate for at least 2 weeks before being moved to environmental chambers that allowed for the ability to control lighting schedules and modified cages that contained a running wheel. Mice were allowed access to food and water ad libitum and maintained under a 12 h light/12 h dark schedule.

DRUGS

JNJ-18038683 (1-Benzyl-3-(4-chlorophenyl)-1,4,5,6,7,8-hexahydropyrazolo[3,4-d]azepine) was synthesized by medicinal chemistry group at Janssen Research and Development, L.L.C. as the citrate salt form. For formulations, 20% (w/v) hydroxypropyl-β-cyclodextrin was used to solubilize the compound and a correction factor of 1.5690 was applied to compensate for the salt form of JNJ-18038683. 8-OH-DPAT ((±)-8-Hydroxy-2-dipropylaminotetralin hydrobromide) (Tocris Biosciences) was formulated in saline. A correction factor of 1.32 was applied to compensate for the hydrobromide salt form of 8-OH-DPAT. Both compounds were injected in a volume of 10 mL/kg body mass. With each compound, the pH of the solution was adjusted to neutral before injection.

EXPERIMENTAL DESIGN

A phase response curve for JNJ-18038683 (10 mg/kg, i.p.) was generated by administering the compound at various times across the circadian cycle (CT2, 6, 10, 14, 18, or 22) to separate groups of mice for each time point. For these studies, a single animal was used in more than one injection condition and at least 2 weeks were given between conditions to prevent carry-over, or interference effects from the previous trial.

To determine if JNJ-18038683 alters the phase advance of wheel running activity induced by the 5-HT1A/7 receptor agonist, 8-OH-DPAT, mice were first randomized into four separate groups. Animals then received an injection of JNJ-18038683 (3 mg/kg, i.p.) or vehicle. Thirty minutes later, mice were further

designated to receive 8-OH-DPAT (3 mg/kg, i.p.,) or corresponding vehicle at CT6 which had been previously reported to result in a phase advance of the onset of locomotor rhythm by 8-OH-DPAT (Horikawa and Shibata, 2004).

To investigate the role of the 5-HT7 receptor on photic induced phase shifts, JNJ-18036863 (10 mg/kg, s.c.) or vehicle was administered 30 min prior to an acute light pulse (200 lux, 30 min) that occurred at times that are known to produce a photic phase delay (CT 15) or advance (CT 22) of the onset of wheel running activity. As a control for the light pulse, mice were placed in the chamber but not exposed to light. For each treatment, separate groups of mice were used.

WHEEL RUNNING ACTIVITY RECORDING AND ANALYSIS

Wheel running activity was recorded by a magnetic switch located on the wheel that transferred each revolution as an event to an IBM compatible computer in 5 min bins using ClockLab software. Following at least a one-week acclimation period to adjust to the running wheels and novel cage, mice were released into constant darkness to assess free-running conditions and the onset of activity was monitored. On designated experimental days, the onset of activity (designated by convention as CT 12) was calculated based on the free running phase advance of activity of the previous 7 days. All timing for injections and light exposure was predicated by the calculation of CT 12 for each animal. Wheel running activity was monitored for at least 1 week following treatment or corresponding control conditions for changes to the onset of activity. A phase shift was calculated using ClockLab software and defined as the difference in the onset of activity after treatment vs. onset of activity prior to treatment. Data are expressed as mean ± S.E.M. To determine if JNJ-18038683 resulted in a significant phase shift at various time points along the phase response curve, an unpaired *t*-test (Vehicle vs. JNJ-18038683 treated animals) was executed. A one factor, four-level ANOVA followed by *post hoc* analysis was performed for each of the remaining two studies to determine if the phase shift was significant with the four treatment groups ($p < 0.05$). For visualization, a phase advance is denoted as a positive value on the bar graph while a phase delay is graphed as a negative value.

RESULTS

PHASE RESPONSE CURVE WITH JNJ-18038683

To determine the effects of the pharmacological blockade of the 5-HT7 receptor by JNJ-18038683 (10 mg/kg, i.p.), the compound was administered at various circadian times throughout the subjective circadian cycle (CT 2, 6, 10, 14, 18, 22) and the resulting phase shift was calculated post administration and compared to vehicle. At all times tested, JNJ-18038683 failed to elicit a phase shift as determined by unpaired *t*-test (**Table 1**). In a separate study, negative results were also obtained following the administration of a higher dose of JNJ-18038683 (20 mg/kg, i.p.) (data not shown).

NON-PHOTIC REGULATION OF CIRCADIAN RHYTHMS BY THE 5-HT7 RECEPTOR

We then tested whether the 5-HT7 receptor antagonist JNJ-18038683 would attenuate the phase advance of wheel running locomotor activity elicited by the 5-HT1A/7 receptor agonist 8-OH-DPAT. Administration of the vehicle for JNJ-18038683 followed by 8-OH-DPAT resulted in a robust phase advance of the onset of locomotor activity during constant dark conditions (37.0 ± 6.3 min, $F_{(3,58)} = 18.49$ $p < 0.0001$, one-way ANOVA, Tukey's *post hoc* analysis) when compared to the other three treatment groups (Vehicle + Vehicle: −5.4 ± 4.1 min, JNJ-18038683 + Vehicle −0.9 ± 3.6 min, JNJ-18038683 + 8-OH-DPAT: −2.7 ± 3.0 min) (**Figure 1**). Comparable to what had been measured during the generation of the phase response curve, the administration of JNJ-18038683 in conjunction with the vehicle for 8-OH-DPAT did not produce any phase shift at CT 6. The administration of the 5-HT7 receptor antagonist JNJ-18038683 completely blocked the phase advance produced by 8-OH-DPAT (**Figure 1**).

PHOTIC REGULATION OF CIRCADIAN RHYTHMS BY THE 5-HT7 RECEPTOR

To determine the effects of pharmacological blockade of the 5-HT7 receptor on photic control of circadian rhythms,

Table 1 | Phase response curve following the administration of the 5-HT7 receptor antagonist JNJ-18038683 (10 mg/kg, i.p.) at various circadian times.

	Phase shift (min.)	
CT	Vehicle	JNJ-18038683
2	0.0 ± 0.0	0.25 ± 0.25
6	0.0 ± 0.0	−0.15 ± 0.10
10	−0.20 ± 0.10	−0.29 ± 0.20
14	0.0 ± 0.0	−0.08 ± 0.08
18	0.0 ± 0.0	0.0 ± 0.0
22	0.0 ± 0.0	0.0 ± 0.0

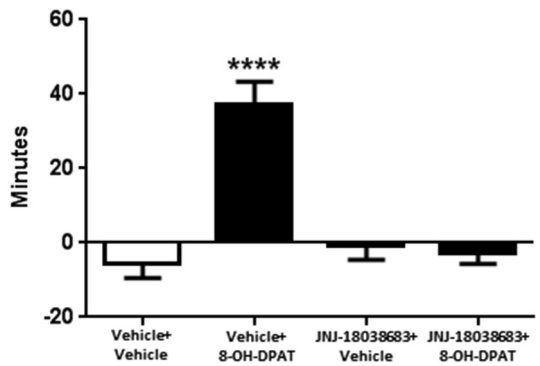

FIGURE 1 | Phase shifting effects of JNJ-18038683 (3 mg/kg. i.p.) and 8-OH-DPAT (3 mg/kg, i.p.) on the circadian rhythms of wheel running activity in mice. Mice were randomly divided to receive one of four treatments: Vehicle + Vehicle ($n = 14$), Vehicle + 8-OH-DPAT ($n = 21$), JNJ-18038683 + Vehicle ($n = 11$), and JNJ-18038683 + 8-OH-DPAT ($n = 16$). After treatment, phase shifts of locomotor activity were calculated based upon the onset of activity following treatment compared to prior treatment. Results are expressed as mean ± S.E.M. and a one-way ANOVA followed by a Tukey's *post hoc* test was performed to determine if a result was significant (****$p < 0.0001$ vs. Vehicle + Vehicle, JNJ-18038683 + Vehicle, and JNJ-18038683 + 8-OH-DPAT).

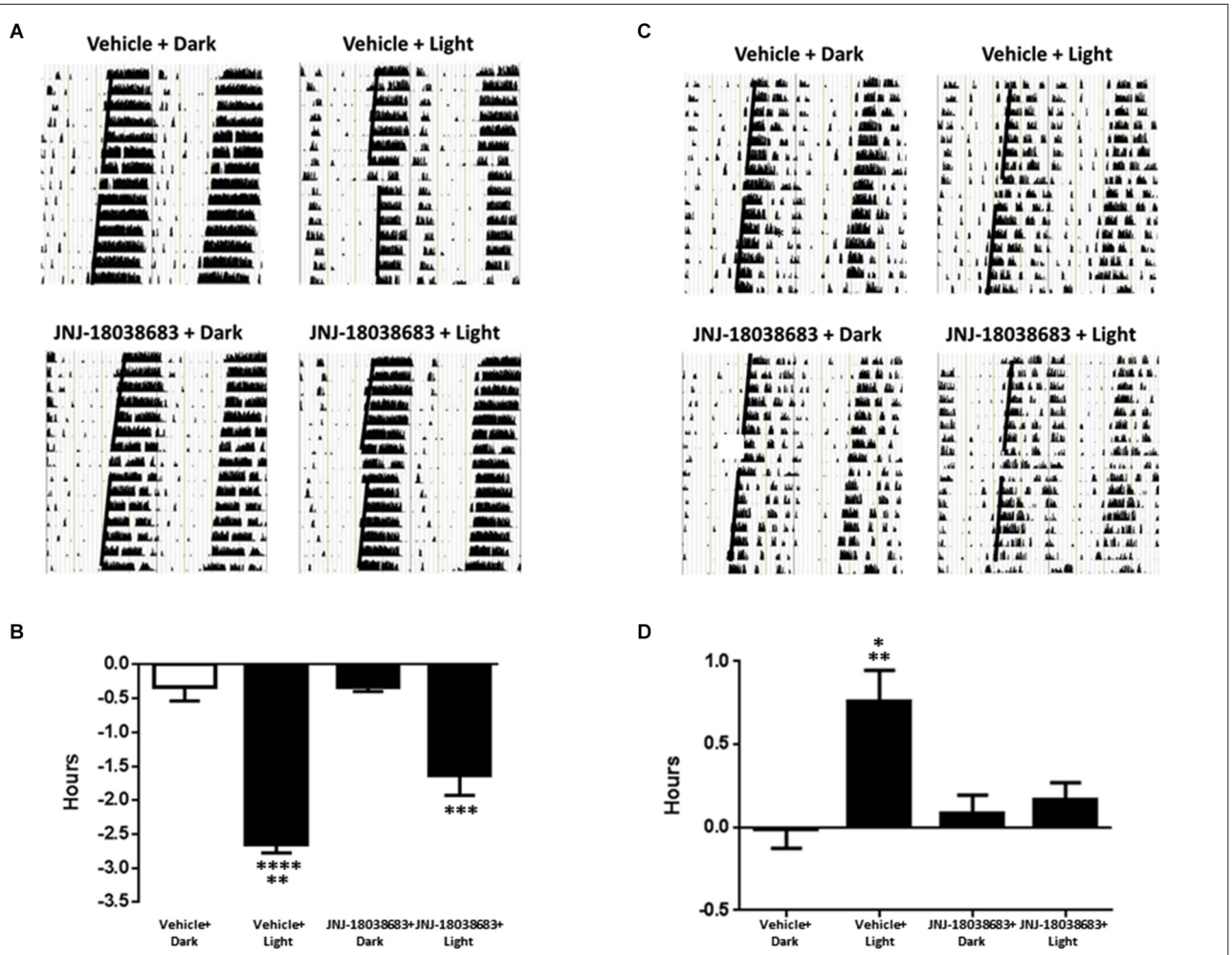

FIGURE 2 | The impact of the 5-HT7 receptor antagonist JNJ-18038683 (10 mg/kg, s.c.) on light induced phase shifts. Representative actigrams and resulting phase shifts following administration of Vehicle/JNJ-18038683 or dark/light pulse on the circadian rhythms of wheel running activity in mice. Mice were randomly divided to receive one of four treatments: Vehicle + Dark (n = 5), Vehicle + Light Pulse (n = 6), JNJ-18038683 + Dark (n = 6), and JNJ-18038683 + Light Pulse (n = 5, CT 15; n = 6, CT 22). The light pulse was administered at times known to phase delay (CT15) **(A,B)** or advance (CT22) **(C,D)** the onset of wheel running activity in mice. After treatment, phase shifts of locomotor activity (onsets of activity indicated by bold black line) were calculated based upon the onset of activity following treatment compared to prior treatment. Results are expressed as mean ± S.E.M. and a one-way ANOVA followed by a Tukey's *post hoc* test was performed to determine if a result was significant (Phase Delay—$^{**}p < 0.01$ vs. JNJ-18038683 + Light, $^{***}p < 0.001$ vs. JNJ-18038683 + Dark, $^{****}p < 0.0001$ vs. Vehicle + Dark and JNJ-18038683 + Dark; Phase Advance—$^{*}p < 0.05$ vs. JNJ-18038683 + Light, $^{**}p < 0.01$ vs. Vehicle + Dark and JNJ-18038683 + Dark).

JNJ-18038683 was administered prior to a light pulse and the potential alterations in the onset of mouse wheel running activity was analyzed. In vehicle treated animals, light exposure resulted in a significant phase shift in the onset of activity at both times tested (CT 15: −2.65 ± 0.12 h: $F_{(3,18)}$ = 42.37; CT22: 0.76 ± 0.19 h: $F_{(3,19)}$ = 6.757 one way ANOVA, Tukey's *post hoc* analysis) (**Figures 2A–D**). Administration of JNJ-18038683 attenuated the light-induced phase delay (−1.64 ± 0.29 h) (**Figures 2C,D**) while the resulting phase advance following light exposure at CT 22 was completely blocked by the compound (**Figures 2A,B**).

DISCUSSION

The present investigation examined the impact of the 5-HT7 receptor antagonist JNJ-18038683 on non-photic and photic regulation of the circadian rhythm of wheel running activity in mice. Results reported from these studies demonstrate that the compound did not exert direct non-photic or photic phase resetting properties by itself. However, JNJ-18038683 was able to block the non-photic phase advance of wheel running activity induced by the 5-HT1A/7 receptor agonist 8-OH-DPAT. In addition, JNJ-18038683 completely blocked the phase advance and significantly attenuated the phase delay of wheel running activity induced by a light pulse.

PHASE RESPONSE CURVE OF JNJ-18038683

To determine if JNJ-18038683 exerts direct non-photic or photic phase resetting effects in free running mice, the compound was administered at specified times across the circadian cycle. At all

times tested, JNJ-18038683 failed to elicit a phase shift when compared to vehicle treated mice. The lack of effect with JNJ-18038683 on phase shifts in mice was similar to what has been reported for the 5-HT2/7 receptor antagonist, ritanserin and a more selective 5-HT7 receptor antagonist SB-269970 in hamsters and rats (Antle et al., 1998; Duncan et al., 2004; Westrich et al., 2013). While antagonists for the 5-HT7 receptor do not modulate non-photic regulation of circadian rhythms, agonists for this receptor (8-OH-DPAT and LP-211) are capable of shifting the onset of wheel running activity in constant conditions (Horikawa and Shibata, 2004; Adriani et al., 2012). In addition, fluoxetine that inhibits the reuptake of serotonin and therefore, universally activates the serotonergic system has been shown to induce non-photic phase shifts (Cuesta et al., 2008, 2009). Therefore, global activation of the serotonergic system or direct pharmacological activation of the 5-HT7 receptor may be needed to induce photic or non-photic phase shifts.

NON-PHOTIC REGULATION OF CIRCADIAN RHYTHMS BY THE 5-HT7 RECEPTOR

The second study was designed to determine if the acute pharmacological blockade of the 5-HT7 receptor with the selective antagonist JNJ-18038683 impacted the phase advance of wheel running activity induced by 8-OH-DPAT in mice. The timing of 8-OH-DPAT administration was based on previous studies that demonstrated a robust phase advance when the compound was injected at CT 6 (Horikawa and Shibata, 2004). When JNJ-18038683 was administered before 8-OH-DPAT in the current study, the phase advance induced by the agonist was completely blocked demonstrating the impact of the 5-HT7 receptor on non-photic phase resetting of circadian rhythms. Since 8-OH-DPAT is an agonist for both the 5-HT1 and 5-HT7 receptors, future studies will utilize selective agonists for the 5-HT7 receptor (i.e., LP-44 or LP-211) to further elucidate the role of the 5-HT7 receptor on non-photic regulation of circadian rhythms.

Earlier *in vivo* and *in vitro* studies from other labs have provided some insight into a mechanism by which the 5-HT7 receptor regulates non-photic control of circadian rhythms. During mid-subjective day, micro-injection of 8-OH-DPAT or 5-Carboxamidotryptamine (5-CT) into the dorsal raphe nucleus activates 5-HT7 receptors and subsequently phase advances the circadian rhythms of wheel running activity in hamsters (Mintz et al., 1997; Duncan et al., 2004; Duncan and Davis, 2005). These phase shifts were blocked by the administration of the selective 5-HT7 receptor antagonist SB-269970 (Duncan et al., 2004). Further investigation delineated this model to show that the activation of the 5-HT7 receptor inhibits glutamate release within the dorsal raphe nucleus resulting in the inhibition of the release of GABA from interneurons which subsequently relieves the inhibition on serotonergic neurons located within the dorsal or medial raphe nucleus (Glass et al., 2003; Harsing et al., 2004; Duncan and Congleton, 2010). The subsequent release of serotonin onto the SCN results in non-photic phase modulation (Duncan and Congleton, 2010). Therefore, administration of JNJ-18038683 may be acting on 5-HT7 receptors within the dorsal raphe nucleus to inhibit glutamate release and thus blocking the non-photic phase shifts induced by 8-OH-DPAT. However, further studies are needed to define specific pathways in which the 5-HT7 receptor modulates non-photic circadian re-setting since the receptor is localized not only within the dorsal/median raphe nucleus to modulate GABA release, but also within the primary circadian oscillator of the SCN and other brain regions known to influence circadian rhythms such as the thalamus that could potentially impact NPY release which has been shown to alter non-photic phase shifts (Mrosovsky, 1996; Belenky and Pickard, 2001; Neumaier et al., 2001; Bonaventure et al., 2002; Gamble et al., 2006; Matthys et al., 2011; Hughes and Piggins, 2012).

PHOTIC REGULATION OF CIRCADIAN RHYTHMS BY THE 5-HT7 RECEPTOR

In addition to the non-photic influence of circadian rhythms, serotonin can also alter photic responsiveness of the SCN possibly by acting via the 5-HT7 receptor. Early studies associated the 5-HT7 receptor with changes in the sensitivity of light in SCN neurons. Administration of serotonin, 5-CT, or 8-OH-DPAT reduced the firing of SCN neurons located in the hamster following a light pulse (Ying and Rusak, 1997). This decrease in neuronal firing was subsequently reversed by applying ritanserin or clozapine which are known to antagonize the 5-HT7 receptor but not the 5-HT1A/B/D receptor antagonist, cyanopindolol or the 5-HT1A receptor antagonist WAY-100635 (Ying and Rusak, 1997). In subsequent studies, hypothalamic excitatory post synaptic currents evoked by optic nerve stimulation which mimics a light pulse were reduced when the 5-HT1A/B agonist TFMPP was added to the bath (Smith et al., 2001). The change in these glutamate dependent currents within the hypothalamus were significantly attenuated with ritanserin, thus further implicating a role for the 5-HT7 receptor in photic control of circadian rhythms by modulating glutamate transmission (Smith et al., 2001). The additional findings that TFMPP had little effect in this model in 5-HT1B receptor KO mice and was minimally attenuated with the 5-HT1A receptor antagonist WAY-100635, provide some indirect evidence that the role of the 5-HT7 receptor in modulating photic control of circadian rhythms may be in coordination with other serotonergic receptors and glutamatergic neurotransmission.

Additional studies have investigated a role for the 5-HT7 receptor in the photic control of circadian rhythms by generating a phase response curve of light in 5-HT7 receptor KO and corresponding WT mice (Gardani and Biello, 2008). Overall, the phase shifts induced by the light pulse were comparable in magnitude and direction between WT and 5-HT7 receptor KO mice except at CT 22 (Gardani and Biello, 2008). At this time point, the light pulse resulted in the requisite phase advance in WT mice but a phase delay in the 5-HT7 receptor KO mice (Gardani and Biello, 2008). Interestingly, while the current study measured a significant attenuation of the light induced phase delay at CT 15 with the administration of JNJ-18038683, there was no difference in WT or 5-HT7 receptor KO mouse when the light pulse was administered at CT 14 or 16 which replicated an earlier report (Sprouse et al., 2003). The difference in regards to the light induced phase shift at these earlier time points

between the pharmacological and the genetic deletion of the receptor may be the result of compensatory mechanisms involved in the phase resetting after a light pulse at these earlier time points of the subjective dark phase in the 5-HT7 receptor KO mouse.

Since this is the first report of attenuation of phase shifts following light pulses following the acute pharmacological blockade of the 5-HT7 receptor, additional studies are needed to further investigate the mechanism by which the 5-HT7 receptor is modulating the photic control of circadian rhythms. Results from these studies will provide an explanation as to why pharmacological blockade of the 5-HT7 receptor resulted in diminished photic induced phase shifts as opposed to an enhancement given the finding that activation of the 5-HT7 receptor inhibits the RHT input to the SCN by decreasing glutamate (Ying and Rusak, 1997; Smith et al., 2001). In addition, since antagonists selective for the 5-HT7 receptor lack direct photic-like phase shifting effects by themselves, the actions of these 5-HT7 receptor antagonists are probably due to the coordination with other serotonin receptors including the 5-HT1A and 5-HT1B receptors (Ying and Rusak, 1997; Rea, 1998; Smith et al., 2001).

Similar to our findings that JNJ-18038683 diminished photic induced phase resetting, pharmacological agents that are known to activate the serotonin system such as SSRIs (fluoxetine, citalopram, fluvoxamine, and paroxetine) also reduce the phase shift induced by a light pulse in the hamster (Gannon and Millan, 2007). Interestingly, fluoxetine potentiates the light induced phase shift in the diurnal species *Arvicanthis ansorgei* (Cuesta et al., 2008). These contrasting effects between nocturnal and diurnal species may be explained by the differential regulation of brain concentrations of serotonin corresponding to arousal state and also may be due to the changes in expression of circadian controlled genes following a light pulse in nocturnal and diurnal animals (Faradji et al., 1983; Poncet et al., 1993; Rea et al., 1994; Weber et al., 1998; Cuesta et al., 2008). While the current study did not measure the changes in the expression of such genes in response to pharmacological manipulation of the 5-HT7 receptor, Westrich and colleagues reported that the *in vitro* period as measured by a luciferase reporter linked to the PER2 gene, can be shortened by the 5-HT7 receptor agonist AS-19 and subsequently blocked by the 5-HT7 receptor antagonist SB-269970 (Westrich et al., 2013).

Due to the possible potentiation of the effects of light in diurnal species such as humans, 5-HT7 receptor antagonists may provide an interesting adjunctive therapy for the antidepressant effects associated with bright light therapy. The idea of adjunctive therapy with a 5-HT7 receptor antagonist arose from earlier studies in which JNJ-18038683 or SB-269970 enhanced the antidepressant-like effects of an SSRI in pre-clinical studies (Bonaventure et al., 2007, 2012; Wesołowska et al., 2007). When JNJ-18038683 was administered in combination with citalopram, there was an enhancement of serotonin transmission, antidepressant-like behavior, and REM sleep suppression induced by the SSRI in rodents (Bonaventure et al., 2012). In another study, Westrich et al. demonstrated that the administration of escitalopram or the 5-HT7 receptor antagonist SB-269970 was ineffective at phase shifting wheel running activity in rats, however, the co-administration of escitalopram with SB-269970 resulted in phase delays in rodent wheel running (Westrich et al., 2013). In addition to adjunctive therapy with an SSRI, a 5-HT7 receptor antagonist may also provide useful adjunctive therapy to bright light exposure in humans. While bright light therapy has been used for several decades as a stand-alone antidepressant therapy for seasonal affective disorder (Pail et al., 2011), it has also been used in conjunction with SSRI for major depressive disorder to bolster and hasten the antidepressant properties of SSRIs since the onset of mood improvement may take several weeks and also to increase the responsiveness in those individuals who are resistant to treatment with SSRIs by themselves (Benedetti et al., 2003; Blier, 2003; Martiny, 2004; Martiny et al., 2004; Wirz-Justice et al., 2005). There is clinical evidence that bright light therapy with sertraline can be therapeutically beneficial as an adjunct therapy for depression resulting in significant reductions in the HAM-D scale of depression (Martiny et al., 2005a,b). In addition, combining bright light therapy with citalopram has been shown to accelerate the onset of the anti-depressant properties of the SSRI (Benedetti et al., 2003). Therefore, combining bright light therapy with JNJ-18038683 may enhance the antidepressant effects of the light exposure in humans. Findings from these studies help provide insight into the translational therapeutic benefits of combining a 5-HT7 receptor antagonist with additional antidepressant modalities including bright light therapy that would correct the various circadian disruptions that are commonly associated with depression.

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5-HT$_7$ receptor stimulation and blockade: a therapeutic paradox about memory formation and amnesia

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OVERVIEW

Mammalian memory involves multiple brain areas and neurotransmitter systems; both at the receptor and post-receptor level (Meneses, 2014a,b). Investigations of serotonin (5-hydroxytryptamine, 5-HT) involvement in memory (Meneses, 2013) have been significantly enhanced by the identification, classification, and cloning of multiple receptors (Hoyer et al., 1994, 2002) and studies at the post-receptor level (Raymond et al., 2001). Emergent investigation of 5-HT$_7$ receptor is growing but contradictory. Hence, major current questions include (1) the paradox that 5-HT$_7$ receptor agonists and antagonists in behavioral memory tasks are revealing promnesic and antiamnesic effects; (2) 5-HT$_7$ and 5-HT$_{1A}$ receptors interaction (and other 5-HT receptors), and neural markers associated to these cognitive processes and individual differences. In this work recent data are briefly revised.

5-HT$_7$ RECEPTOR STIMULATION

Freret et al. (2014) reported that with a 2-h delay, (post-training) administration of the 5-HT$_7$ receptor antagonist SB-269970 (3 and 10 mg/kg, sc) impaired the discrimination of the novel object; but with a 4-h delay, while control mice were not able to discriminate the novel object, mice treated with the agonist 5-carboxamidotryptamine (5-CT; displaying affinity for several receptors including 5-HT$_7$; Hoyer et al., 1994) showed a significant discrimination. This promnesic effect was blocked by SB-269970, but not by WAY-100135 or GR-127935 (5-HT$_{1A}$ or 5-HT$_{1B/1D}$ receptor antagonists, respectively; Hoyer et al., 1994). Freret et al. (2014) conclude that 5-HT$_7$ receptor tonically modulates cognitive processes involved in consolidation performances in object recognition and therefore, it could be a promising target to treat memory dysfunctions (especially episodically related deficits) or pathological aging. Notably, Stahl (2010) suggests that 5-HT$_7$ receptor (blockade) as a novel therapeutic target for antidepressant and pro-cognitive effects. Nikiforuk et al. (2013) also conclude that antagonism of 5-HT$_7$ receptor may represent a useful pharmacological approach in the treatment of cognitive deficits and some negative schizophrenia symptoms. Also, Tajiri et al. (2012) and Gasbarri and Pompili (2014) propose that the 5-HT$_7$ receptor is a rational target for the treatment of psychiatric disorders. Certainly, more research is necessary about 5-HT$_7$ receptor, its functional complexity in memory formation and abnormal memory as well timing of drug administration (McGaugh, 1989; Monleón et al., 2008).

Pre-training elevation of 5-HT by the selective serotonin reuptake inhibitor (SSRI) fluoxetine had no effect by itself, but facilitated passive avoidance when combined with the 5-HT$_{1A}$ receptor antagonist NAD-299 and this facilitation was blocked by SB-269970 (Eriksson et al., 2012). Likewise Eriksson et al. (2012) reported that a reduced activation of the 5-HT$_{1A}$ receptor was resulting in enhanced stimulation of the 5-HT$_7$ receptor but 5-HT$_7$ receptor agonists LP-44 or AS19 failed to facilitate passive avoidance performance; and according with these authors LP-44 and AS19 have low efficacy to stimulate protein phosphorylation of 5-HT$_7$ receptor-activated signaling cascades. Notably, in the mutant mouse brain (lacking pituitary adenylate cyclase, an experimental mouse model for psychiatric disorders) 5-HT$_7$ protein expression did not differ from wild-type mice but in primary embryonic hippocampal neurons AS-19 increased neurite length and number (Tajiri et al., 2012). Likewise, LP-211 and 8-OHDPAT affect neurites in embryonic cultures (Speranza et al., 2013). Certainly, Eriksson et al. (2012) propose that retention (in passive avoidance) is mediated through hippocampal 5-HT$_{1A}$ receptor activation, while the 5-HT$_7$ receptor appears to facilitate memory processes in a broader cortico-limbic network and not the hippocampus alone. Notably, LP-211 rescued diverse defective performances, including memory in the novelty preference task as well as the abnormal activation of PAK and cofilin (key regulators of actin cytoskeleton dynamics) and of the ribosomal protein (rp) S6, whose reduced activation in MECP2 mutant neurons is responsible for the altered protein translational control (De Filippis et al., 2014). De Filippis et al. (2014) indicate that pharmacological targeting of 5-HT$_7$ receptor improves specific behavioral and molecular manifestations and these data are a first step toward the validation of an innovative systemic treatment to disorders associated with intellectual disability.

Importantly, either AS-19 or LP-211 or the dual 5-HT$_{1A/7}$ receptor agonist, 8-OHDPAT, facilitated memory consolidation in an associative memory task and increased cAMP production (Pérez-García et al., 2006; Meneses, 2013; Meneses et al., 2014) which were reversed

by SB-269970. Certainly, an association between memory and cAMP production exists (Kandel, 2001; Izquierdo et al., 2006). It should be noted however that memory formation in an autoshaping learning (Pérez-García and Meneses, 2008) showed that cAMP production was decreased by 8-OHDPAT if memory was improving but the opposite occurred in absence of memory. In autoshaping task, trained animals are food-restricted (at 85% of *ad-libitum)*, receiving one autoshaping training and are tested at 1.5 h for short-term memory (STM) and at 24 and 48 h for consolidation of long-term memory (LTM) (Meneses, 2013). Why 8-OHDPAT improved memory (consolidation, 48 h) and increased cAMP in cortex and hippocampus (Manuel-Apolinar and Meneses, 2004) or decremented (Pérez-García and Meneses, 2008) hippocampal cAMP production? Importantly, the new 5-HT$_7$ receptor agonist LP-211 did not affect STM; nonetheless, at 0.5 and 1.0 mg/kg it improved LTM. The 5-HT$_7$ receptor antagonist SB-269970 (10.0 mg/kg) alone had not effect but it reversed the LP-211 (1.0 mg/kg) LTM facilitation. The scopolamine (0.2 mg/kg) induced-decrement in CR was accompanied by significant increased cAMP production. Scopolamine-induced amnesia and increments in cAMP, are significantly but not completely reversed by LP-211 (Meneses et al., 2014). Hence, prefrontal cortex, cAMP production and improved memory formation seem to be associated; however, this association is complex and dependent on the basal level (e.g., prevalent expression of either of 5-HT$_{1A}$ or 5-HT$_7$ receptors). For instance, the time-course (0–120 h) of autoshaped responses revealed progressive performance and mRNA 5-HT$_{1A}$ or 5-HT$_7$ receptors are monotonically augmented or declined in prefrontal cortex, hippocampus and raphe nuclei, respectively (Perez-Garcia and Meneses, 2009). Moreover, 5-HT$_{1A}$ receptor is increased, whereas 5-HT$_7$ receptor levels are decreased by aging (Saroja et al., 2014). Also, they showed significant correlation with the time spent in target quadrant; hence according with Saroja et al. (2014) these are two key parameter of memory retrieval which in turn unambiguously links the serotonergic receptor system to spatial memory performance.

5-HT$_7$ RECEPTOR BLOCKADE: COGNITIVE DEMAND OR MEMORY IMPAIRED

Gasbarri et al. (2008) showed that SB-269970 improved memory, decreasing the number of errors in test phase and, thus, affecting reference memory, while no effects in working memory; postulating that 5-HT$_7$ receptor blockade had procognitive effect, when the learning task implicated a high degree of difficulty. In addition, the 5-HT$_7$ receptor antagonists, SB-269970 or DR 4004 alone had no effect but reversed amnesia induced by scopolamine and dizocilpine (Meneses, 2004). Hence, 5-HT$_7$ receptor antagonism plays an important role under poor memory or when the learning or memory is complex.

5-HT$_7$ BLOCKADE AND PROMISCUOUS AFFINITY

In recent time, it has become evident that 5-HT$_7$ blockade and drugs displaying promiscuous affinity have interesting effects. For instance, lurasidone (affinity for several receptors including 5-HT$_7$) and the selective 5-HT$_7$ receptor antagonist, SB-656104-A improved learning and memory deficits by dizocilpine (or MK-801), in the rat passive avoidance test, and AS-19 (3 mg/kg) completely blocked the attenuating effects of lurasidone (3 mg/kg); AS-19 (1–10 mg/kg) pre-training administration had no effects (Horisawa et al., 2013). SB-269970 (30 but not 10 mg/kg) pre-training administration produces both anti-psychotic-like (amphetamine- or phencyclidine-induced hyperactivity tests) and pro-cognitive (novel object discrimination test) activity in preclinical animal models (Waters et al., 2012); these authors conclude that SB-269970 is more a potent inverse agonist than SB-258741, which might be a potential explanation for the conflicting profiles *in vivo* (for references see Waters et al., 2012). Importantly, Huang et al. (2014) reported that 5-HT$_{1A}$ and 5-HT$_7$ receptors contribute to lurasidone-induced dopamine efflux, concluding that, at least partially, 5-HT$_{1A}$ agonist and 5-HT$_7$ antagonist properties may contribute to reversing schizophrenia-like effects. Chronic stress impaired performance on the extra-dimensional (ED) set-shifting stage of the frontal-dependent attentional set-shifting task and amisulpride (3 mg/kg) before testing reversed this restraint-induced cognitive inflexibility and improved ED performance of the unstressed control group (Nikiforuk and Popik, 2013). AS-19 (10 mg/kg) pre-training alone had no effect but abolished the pro-cognitive efficacy of amisulpride (Nikiforuk and Popik, 2013). Nikiforuk et al. (2013) also reported that acute administration of SB-269970 (1 mg/kg) or amisulpride (3 mg/kg) ameliorated ketamine-induced cognitive inflexibility and novel object recognition deficit in rats; both compounds were also effective in attenuating ketamine-evoked disruption of social interactions. In contrast, neither SB-269970 nor amisulpride affected ketamine-disrupted prepulse inhibition; ketamine is a glutamatergic antagonist (Neill et al., 2010). Importantly, in contrast to the negative regulatory effects of long-term activation of 5-HT$_7$ receptors on NMDA receptor signaling (*in vitro*) acute activation of 5-HT$_7$ receptors promotes NMDA receptor activity (Vasefi et al., 2013). Hence, these findings highlight the potential for temporally differential regulation of NMDA receptors by the 5-HT$_7$ receptor. While some inconsistencies might be related to the opposite action exerted by 5-HT$_{1A}$ and 5-HT$_7$ receptors over cAMP production (Renner et al., 2012; Meneses, 2014a,b); certainly, in different protocols of training/testing (memory consolidation vs. STM and LTM), various brain areas and neurotransmission systems interaction might be also implicated.

5-HT$_{1A}$ AND 5-HT$_7$ RECEPTORS

The functional significance of 5-HT$_{1A}$ and 5-HT$_7$ receptors dimerization is has been revised (Matthys et al., 2011; Gellynck et al., 2013; Herrick-Davis, 2013); indicating that it differentially regulates receptor signaling and trafficking (Renner et al., 2012). But still we do not know the implications of this in memory formation and amnesic conditions; hence, the study of signaling associated to 5-HT$_7$ receptor in memory formation, amnesia and forgetting might provide significant insights (Meneses, 2014a,b). For instance, while the association of 5-HT$_7$

receptor stimulation, improved memory and increased cAMP seems to be reliable findings; certainly factors such as differential regulation of hippocampal expression as well as individual differences might be important (see Meneses, 2013). In addition, a biphasic and brain-region selective down-regulation of cAMP concentrations is observed supporting object recognition in the rat (Hotte et al., 2012). Wang et al. (2013) have noted that while it is relatively well established that cAMP signaling is involved in the mediation of memory, the reports on its role to date are inconsistent. One hypothesis is that overactive cAMP signaling impairs working memory in the aged prefrontal cortex (PFC) or activation of the cAMP signaling in the frontal cortex is necessary for working memory; the explanations for this discrepancy may include: (i) activation of cAMP signaling within the PFC and an inverted U-shape dose-response on working memory and memory optimum range of cAMP rather than an overmuch or scanty production; (ii) the continuous and dynamic updating of cAMP levels occurs at the different time-course of memory formation; (iii) cAMP activation might be beneficial for working memory under conditions that require hippocampal–PFC interactions (Wang et al., 2013). Given the complexity of cAMP-dependent responses; hence studies of brain areas and individual differences remain to be reported at both behavioral and cellular levels (see Wang et al., 2013).

Evidence exists of individual differences regarding memory (Ballaz et al., 2007a,b; Fitzpatrick et al., 2013; Flagel et al., 2014) and forgetting (Tellez et al., 2012); hence, it seems reasonable to suggest that in autoshaping (or sign-tracking; see Meneses, 2003) the increment (Manuel-Apolinar and Meneses, 2004) or decrement (Pérez-García and Meneses, 2008) cAMP production might be involving individual differences (e.g., Meneses, 2014a,b). Notably, individual variation in the magnitude and influence of cue reactivity over behavior in humans and animals suggest that cue-reactive individuals may be at greater risk for the progression to addiction and/or relapse (Anastasio et al., 2014).

Also, an important implication is that 5-HT$_7$ (and/or 5-HT$_{1A}$) receptors stimulation increased or decreased cAMP production (e.g., Hoyer et al., 1994) and improved memory (Meneses et al., 2014). Likewise, evidence indicates that 5-HT$_7$ splice variants constitutively activate adenylyl cyclase (Leopoldo et al., 2011). It should be crucial to confirm if memory formation, amnesia, or forgetting by themselves and/or plus drugs modify adenylyl cyclase.

Hence, (1) memory requires restricted or selective cAMP production (Pérez-García and Meneses, 2008; Meneses, 2013); (2) a major and consistent emerging finding is that 5-HT$_7$ receptor stimulation seems to facilitate memory formation and reverse memory impairment; (3) expression of 5-HT$_7$ (and 5-HT$_{1A}$) receptors are accompanying memory; (4) the 5-HT$_7$ antagonism alone had no effect but reversed memory deficits. Notably, a combination of neural and cognitive processes may contribute an early and specific marker of disorders associated to dysfunctional cognitive skills or memory in psychiatric disorders (Millan et al., 2012), including Alzheimer's disease progression (Ibanez and Parra, 2014).

CONCLUSIONS

Behavioral and molecular studies may be particularly insightful and timely in view of the apparently contradictory notion that either 5-HT$_{1A}$ or 5-HT$_7$ receptor agonists or antagonists are useful in the treatment of learning and memory disorders. Also, the distinction about normal memory or impaired memory, timing of drug administration and individual differences are providing important insights about 5-HT$_{1A}$ or 5-HT$_7$ receptors stimulation and blockade.

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Spatial memory deficit across aging: current insights of the role of 5-HT$_7$ receptors

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Elderly persons often face biological, psychological or social changes over time that may cause discomfort or morbidity. While some cognitive domains remain stable over time, others undergo a decline. Spatial navigation is a complex cognitive function essential for independence, safety and quality of life. While egocentric (body-centered) navigation is quite preserved during aging, allocentric (externally-centered) navigation—based on a cognitive map using distant landmarks—declines with age. Recent preclinical studies showed that serotonergic 5-HT$_7$ receptors are localized in brain regions associated with allocentric spatial navigation processing. Behavioral assessments with pharmacological or genetic tools have confirmed the role of 5-HT$_7$ receptors in allocentric navigation. Moreover, few data suggested a selective age-related decrease in the expression of 5-HT$_7$ receptors in pivotal brain structures implicated in allocentric navigation such as the hippocampal CA3 region. We aim to provide a short overview of the potential role of 5-HT$_7$ receptors in spatial navigation, and to argue for their interests as therapeutic targets against age-related cognitive decline.

Keywords: spatial cognition, Alzheimer's disease, serotonin, aging, 5-HT$_7$R

The world's population is aging at an unprecedented rate and constitutes a significant public health issue. Aging is associated with the decline in selective aspects of cognitive performance, together with brain functional and anatomical changes. Among cognitive functions that decline with age, spatial navigation is impaired after the age of 60, with an acceleration in decline after 70 (Barrash, 1994). Spatial navigation is a complex cognitive ability that is essential for independence, safety and quality of life. Spatial navigation capacities have also recently attracted attention in the field of neurodegenerative disorders, especially Alzheimer's disease (AD; Lithfous et al., 2013). Indeed, from a clinical point of view, diagnosis of AD in very early stages appears to be a crucial challenge to optimize therapeutic management. The modulation of the last discovered serotonin receptors, 5-hydroxytryptamine receptors type 7 or 5-HT$_7$R, might be a promising therapeutic approach.

EGOCENTRIC VS. ALLOCENTRIC SPATIAL STRATEGY SHIFT ACROSS AGING

Spatial navigation refers to the process of determining and maintaining a course or trajectory to a goal location (Franz and Mallot, 2000). Two main kinds of strategies are commonly distinguished, depending on the frame of reference used to encode location: egocentric vs. allocentric (Zaehle et al., 2007). When using an egocentric frame of reference, spatial information is encoded from the viewpoint of the individual: it corresponds to self-centered navigation. For example, an egocentric strategy can refer to the association between a particular landmark and left or right body turn in response to this landmark (e.g., turn right at the bookshop, then turn left at the museum). Allocentric navigation is based on non-self-centered maps, i.e., independent of the individual position (O'Keefe and Nadel, 1978). The individual memorizes spatial relationships between landmarks, such as relative direction, angle and distances (Dolins and Mitchell, 2010). Unlike egocentric strategies, allocentric strategies enable an individual to plan novel routes during navigation.

Among the numerous experimental procedures designed to assess spatial learning abilities, most of them allow only one kind of strategy to be used. However, some paradigms offer the opportunity to use either an egocentric or an allocentric strategy (Paul et al., 2009). Several studies have shown that even though individuals spontaneously select one strategy, they are still able to switch when necessary (*humans*: Iaria et al., 2003; Etchamendy and Bohbot, 2007; Iglói et al., 2009; *rodents*: Gibson and Shettleworth, 2005). Thus, this coexistence of strategies allows individuals to adapt themselves to environmental constraints, by shifting for instance from one strategy to another when some spatial information becomes unreliable (Healy, 1998; Shettleworth, 2009).

Functional neuroimaging and neuropsychological studies have provided evidence that the hippocampus and para-hippocampal areas are critically involved when using an allocentric frame of reference (Bohbot et al., 1998; Maguire et al., 1998; Moffat et al., 2006). Conversely, right-sided parietal association cortices and subcortical regions, especially the striatum (i.e., caudate

nucleus and putamen), are commonly associated with egocentric strategies (Maguire et al., 1998; Hartley et al., 2003; Iaria et al., 2003; Galati et al., 2010). Indeed, while performing a spatial task that offers the choice between the two strategies, the subjects who preferentially use an allocentric strategy display a high activity in the right hippocampus; whereas higher brain activity is observed in the caudate nucleus when the egocentric strategy was preferentially adopted (Iaria et al., 2003). Similar results have been observed in preclinical studies. Through the use of different experimental approaches (lesional, transient inactivation or pharmacological experiments), animal data suggested that egocentric and allocentric strategies rely on different neural networks in rodents: the dorso-striatal and the hippocampal mnesic systems, respectively (Packard and McGaugh, 1992; Packard, 1999; DeCoteau and Kesner, 2000; Miranda et al., 2006; Burgess, 2008). When performing a behavioral task in which both strategies are efficient, a higher neuronal activity (assessed through phospho-CREB immunoreactivity) is observed in the dentate gyrus, hippocampal CA1 and CA3 of rats preferentially using an allocentric strategy. Conversely, dorso-lateral and dorso-medial striatum are more activated in rats preferentially using an egocentric strategy. This parallel functioning is however not always straightforward, since both strategies rely on, at least, some shared brain structures. In humans, the posterior parietal and the frontal cortex appear to be activated both in individuals preferring an egocentric or an allocentric strategy (Iaria et al., 2003). In rodents, although the CA3 is activated when using an allocentric strategy, and conversely the striatum when using an egocentric strategy, the dentate gyrus and mammillary bodies are activated in both cases (Rubio et al., 2012; see **Table 1**).

Advanced age is known to alter spatial cognitive abilities (Moffat, 2009; Klencklen et al., 2012). Thus, healthy elders have poorer performances than their younger counterparts when learning a route (i.e., egocentric strategy) (Moffat et al., 2001; Wiener et al., 2013). Depending on the procedure used, this impairment is not always observed (Etchamendy et al., 2012; Gazova et al., 2013), or sometimes remains quite moderate (Pouliot and Gagnon, 2005). However, when addressing allocentric navigation (using either real or virtual environments), age-related impairments are more systematically observed (Moffat and Resnick, 2002, 2007; Driscoll et al., 2003, 2005; Iaria et al., 2009; Gazova et al., 2013). In addition, compared to children, the proportion of subjects preferentially choosing an allocentric strategy in a spatial task is decreased in elders (Bohbot et al., 2012; Rodgers et al., 2012). Aging seems to specifically impair the ability of switching to an allocentric strategy, when an egocentric strategy becomes unreliable (Harris et al., 2012). All results above are consistent with animal studies, showing age-related impairments when using an allocentric strategy (Gallagher and Pelleymounter, 1988; Begega et al., 2001). Additionally, even if aged mice are still able to use an allocentric strategy, they preferentially use an egocentric strategy when they have the opportunity to choose (Nicolle et al., 2003). According to Klencklen et al., impairments observed in egocentric tasks may be due to deficits in the planning of the pathway, while deficits observed in allocentric tasks may be due to the lower likelihood of elderly people to learn configurational information about the environment (Klencklen et al., 2012).

Several studies have been performed to better understand the neural mechanisms underlying age-related alteration in spatial abilities. From a neuroanatomical point-of-view regarding age-induced changes, a shrinkage of the caudate nucleus as well as a greatest deterioration of the frontal cortex are among the two modifications the most frequently documented and well-admitted (Raz et al., 1997, 2003; Greenberg et al., 2008; Kalpouzos et al., 2009; Walhovd et al., 2011). In regards to the hippocampus, while most studies found a shrinkage with aging (Du et al., 2006), some did not found any (Good et al., 2001). This discrepancy may be due to the sampling method used: e.g., studies undertaken across a wide spread of ages (e.g., from 18 to 79 in Good et al., 2001) vs. key periods of age-related impairments (e.g., from 58 to 87 years old in Du et al., 2006; Kalpouzos et al., 2009). Beyond these differences, it seems that shrinkage is usually observed across aging only when considering the posterior part of the hippocampus. Hippocampal neuronal integrity has also been shown to be altered in elderly subjects (Driscoll et al., 2003), and a reduced resting-state metabolism has been observed (Small et al., 2002). Moreover, a reduced activation of the neural network underlying allocentric strategy was observed in older adults (Moffat et al., 2006). Konishi and Bohbot showed that the volume of gray matter in the hippocampus positively correlates with spontaneous allocentric strategies in the healthy elderly (Konishi and Bohbot, 2013). Structural, neurochemical and functional age-related changes in hippocampus and associated structures may explain why impairments are observed in allocentric strategies in the elderly.

5-HT$_7$ RECEPTORS AND SPATIAL NAVIGATION

The 5-HT$_7$R belong to the superfamily of G-protein-coupled receptors. Since their discovery, almost 20 years ago, 5-HT$_7$R distribution has been accurately investigated both in humans and in non-human species (*human*: Varnäs et al., 2004; *non-human*: To et al., 1995; Waeber and Moskowitz, 1995; Gustafson et al., 1996; Neumaier et al., 2001; Bonaventure et al., 2004). Even though slight differences can emerge according to the protocols or the techniques used—immunohistochemistry, radiolabelling, qPCR, etc. . ., the central distribution of 5-HT$_7$R is generally in accordance within the three mainly studied species (human, rat and guinea pig) (Leopoldo et al., 2011). In the central nervous system, a higher density of 5-HT$_7$R was found in the hippocampus (particularly in the CA3 and the dentate gyrus, and to a lesser extent in the CA1), in the hypothalamus and in the thalamus (particularly within the anterior part). Additionally, 5-HT$_7$R are also broadly—but less densely—distributed in the cortex, especially in the frontal, piriform and cingulate cortices, as well as in the amygdala and the dorsal raphe nucleus. As regards to its expression in basal ganglia nuclei, results show discrepancies across species. In humans, a high concentration is observed in caudate and putamen nuclei, whereas the striatum appears to be a structure with low 5-HT$_7$R expression in rats (Martín-Cora and Pazos, 2004).

Table 1 | Relative abundance of 5-HT$_7$R within brain structures involved in egocentric and/or allocentric spatial strategies, and evolution of 5-HT$_7$R mRNA expression across aging.

Brain structures	5-HT$_7$ receptors		mRNA expression across aging
	Egocentric	Allocentric	
Dorsal striatum	**XX**		NI
Thalamus (anterior nucleus)	X	X	≈
Hypothalamus (mammillary nucleus)	X	X	≈
Amygdala	X		≈
Cerebellum		X	NI
Hippocampal formation			
CA1	X	X	≈
CA3	X	**XX**	↘
DG	X	X	≈
Cortex			
Medial prefrontal	X	X	NI
Frontal		X	NI
Temporal		X	NI
Parietal	X	X	NI
Occipital		X	NI

Relative density of 5-HT$_7$R:

few
medium
many

Abundance of 5-HT$_7$R are displayed in gray-scale. "X" indicates an involvement in navigational strategies, "XX" highlights the main structure involved in either egocentric or allocentric strategy. "NI" = Non Investigated, and "↘"= decrease of 5-HT$_7$R mRNA expression, "≈" = no change. Table has been drawn according to the following publications in animals: (To et al., 1995; Gustafson et al., 1996; Duncan et al., 1999; Yau et al., 1999; Kohen et al., 2000; Begega et al., 2001, 2012; Neumaier et al., 2001; Moffat and Resnick, 2002; Colombo et al., 2003; Bonaventure et al., 2004; Varnäs et al., 2004; Moffat et al., 2006; Duncan and Franklin, 2007; Rubio et al., 2012).

Given their distribution within the central nervous system, several recent experiments support a role for 5-HT$_7$R in learning and memory processes (for review see Meneses, 2013). Most interestingly, it seems that 5-HT$_7$R plays a major role in hippocampus-dependent memory processing (Roberts et al., 2004; Gasbarri et al., 2008; Sarkisyan and Hedlund, 2009), particularly when learning a location is required to solve the task This has notably been observed while using two different paradigms of the object recognition test. Typically, the test consisted in two consecutive sessions separated by an intersession interval, and is carried out in an open-field. During the first session, animal can freely explore two similar objects. During the second session, according to the paradigm used, one of the two objects is either replaced by a new one (this paradigm is called novel object recognition test), or only displaced (this paradigm is called object-place recognition test (Oliveira et al., 2010)). The difference between those two paradigms of recognition task relies on the more pronounced involvement of the hippocampus in the object-place recognition test (Barker and Warburton, 2011). In the "novel" object recognition test, transgenic 5-HT$_7$R knock-out (KO) mice performed similarly to their wild-type siblings (Sarkisyan and Hedlund, 2009). As a contrary, in the object-place recognition test, while the spatial component of the test is more challenging, KO mice displayed a marked impairment of memory performances. Of note, the same hold true if wild mice are administered before the first session, with SB-269970, a selective 5-HT$_7$R antagonist. Indeed, whereas no differences are noticed for the non-spatial paradigm of the test (novel object recognition) when the antagonist is administrated, impairments are observed in the spatial version of the recognition task (Sarkisyan and Hedlund, 2009). Thus, those two results obtained with either KO mice or after administration of 5-HT$_7$R antagonist both argue for a predominant role of 5-HT$_7$R in behavioral tasks requiring animals to learn location of objects in an allocentric frame of reference. Still in the context of pharmacological modulation of 5-HT$_7$R, a sub-chronic treatment during adolescence withLP-211 (a selective 5-HT$_7$R agonist) did not lead to major modifications of performances in the novel object recognition paradigm at adulthood (Canese et al., 2014). Unfortunately, the effect of an acute tonic pharmacological modulation of 5-HT$_7$R has yet not been investigated in the two paradigms of the recognition test. Again, such a result is in favor of an involvement of 5-HT$_7$R in memory processes when spatial learning is required.

Beyond the importance of the spatial component in the behavioral task, the involvement of 5-HT$_7$R could also vary according to the strategy used to perform the test. In fact, KO mice have been tested in the Barnes maze test (Roberts et al., 2004; Sarkisyan and Hedlund, 2009). This dry-land maze consists of a brightly illuminated elevated circular platform (aversive stimulation), with several holes around its perimeter (Barnes, 1979). Each session of the test starts by placing the animal in the middle of the maze. Animals will then try to escape the aversive bright light maze by searching and entering into the box placed underneath one of the holes. Even though egocentric and allocentric strategies are both efficient in this test (Harrison et al., 2006), a recent study has demonstrated that all of the 13 inbred strains of mice tested preferentially used an egocentric strategy to find the platform (O'Leary et al., 2011). In this task, KO mice did no display any spatial memory impairment (Roberts et al., 2004; Sarkisyan and Hedlund, 2009). Thus, this lack of deficit might be explained by

the use of a striatum-dependent egocentric strategy, spared in these KO mice (Sarkisyan and Hedlund, 2009). Besides, it has been elegantly demonstrated that KO mice displayed a clear deficit in allocentric strategy. Indeed, Sarkisyan and Hedlund performed a reversal test a month later during which the location of the escape box was moved 180° away from its original location. As expected, all mice started looking for the escape box at its original quadrant. But, after having explored the now empty hole, the two populations of mice (KO and wild-type) started to explore the maze in a quite different manner. Indeed, while KO mice found themselves close to the starting position, they continued to use the initial pathway to come back to the empty escape box. Such observation led the authors to hypothesize that KO mice may not be able to switch to an allocentric strategy, so keep using an egocentric one, even though it is no longer reliable. Such behavioral impairments observed in KO mice might be related to the absence of expression of 5-HT$_7$R in the CA3 region of the hippocampus and dentate gyrus. Indeed, those two regions are mainly involved in the integration of environmental changes. Besides, in wild type mice, a high density of 5-HT$_7$R is observed in the CA3 region of the hippocampus and dentate gyrus, relative to other brain regions. Thus, the lack of expression of 5-HT$_7$R within those two brain regions in KO mice would have led to an impairment of allocentric strategy. Unfortunately, the authors did not test this hypothesis with another group of wild-type mice within the same experimental paradigm, but with the presence of a wall around the edge of the maze. Such a group is considered as a "negative control". Indeed, in such condition, since no external visual cue is available, animals are forced to use an egocentric strategy at every step of the test.

Finally, the group of Meneses also investigated the involvement of 5-HT$_7$R in another spatial task, namely the radial arm maze (Gasbarri et al., 2008). In this work, authors observed conflicting results to what we previously described. Indeed, they showed that the pharmacological blockade of 5-HT$_7$R (SB-269970) improved spatial learning performances. Thus, the same pharmacological agent (namely SB-269970) has been demonstrated both to improve (Gasbarri et al., 2008) (see above) or to impair spatial memory performances (Sarkisyan and Hedlund, 2009). Roberts and Hedlund have suggested an explanation that allows for overcoming discrepancies (Roberts and Hedlund, 2012). In fact, in the radial arm maze experiment, the animal is always placed at the center of the maze at the beginning of each session. Thus, they suggested that learning the location of the baited-arm through an egocentric strategy (a direct route from the starting position) could be more efficient than the allocentric one. If this is true, then the better performances observed in treated rats may be explained by the fact that SB-269970 would have favored a more rigid strategy (independent of 5-HT$_7$R).

5-HT$_7$R ACROSS AGING: INSIGHT TO A POTENTIAL THERAPEUTIC TARGET?

Surprisingly, only a few studies have examined changes in brain 5-HT$_7$R density during aging, with only five publications from three different research groups available so far. Unfortunately, the results are not straightforward. The first publication comes from the Seckl lab which used a pharmacological model of adrenalectomy in order to mimic accelerated aging in rats (Yau et al., 1997). In this work, the authors observed, through *in situ* hybridization, an increased expression of 5-HT$_7$R mRNA expression in the hippocampal CA3 regions 24 h after the induction of the experimental model (Yau et al., 1997). However, when 2 years later the same group performed the same experiments but in aged rats (22–24 months), no modification was observed in any of the hippocampal sub-regions (Yau et al., 1999). This apparent discrepancy seems thus more related on the relevance, in the context of 5-HT$_7$R, of the pharmacological model used in the first study in order to mimic aging processes. Kohen et al. have also explored changes in 5-HT$_7$R density across aging in three groups of rats, aged either 3, 12 or 24 months, modeling different stages of aging; adult, middle-aged and old, respectively (Kohen et al., 2000). In this work, the authors have studied the subfields of the hippocampus by separating the ventral and dorsal part. In accordance to the work of Yau et al. in aged rats (Yau et al., 1999), the authors did not observe any change in the dorsal CA3 of the hippocampus. However, they noticed an age-related decline in the ventral CA3 part of the hippocampus. This decrease, already marked in the middle-aged group of rats, remained the same in old rats (24 months). Finally, using another animal model (hamsters), Duncan and Franklin also investigated the effect of aging on mRNA expression of 5-HT$_7$R in discrete forebrain and midbrain regions (Duncan and Franklin, 2007). Three age groups of hamsters (3–5, 12–14 and 17–19 months of age) were also studied. Contrary to what was previously observed, this work has not highlighted alteration of mRNA expression across aging in any of the investigated brain regions—including the hippocampus, with the exception of the decrease measured in the cingulate cortex and the paraventricular thalamic nucleus. In a previous study which focused in four brain structures that regulate circadian cycle (namely the suprachiamastic nuclei, the lateral geniculated nuclei, the median raphe nucleus and dorsal raphe nucleus), the same group of researchers also observed a marked decrease in 5-HT$_7$R in the dorsal raphe nucleus (Duncan et al., 1999). As regards to expression of 5-HT$_7$R mRNA in the hippocampus, several explanations can be proposed to explain discrepancies observed in the effects of aging. Indeed, the different animal models used (rats, mice, hamsters) may account for such divergent results, as well as, for instance, the location of the brain slice performed in the different works (highlighting or masking discrete regions of interest), etc... Besides, one may note that when comparing the works of the group of Yau and Kohen, if the whole structure is taken into account, no age-induced modification is observed, whereas a marked decreased of approximately 30% is well observed when focusing in the ventral part of the hippocampus. Considering the role of the CA3 region of the hippocampus in spatial strategy highlighted above, a decreased expression of 5-HT$_7$R in this brain structure could account for impairments of the shift between spatial strategies across aging.

CONCLUSION AND FUTURE DIRECTIONS

Regarding the question of aging-induced deficit in the shift between egocentric and allocentric strategies and the role of

5-HT$_7$R in these impairments, a crucial lack of data makes it difficult to draw conclusions presently. Indeed, all the previously cited studies exploring the changes of 5-HT$_7$R across aging have occurred at the messenger RNA level; but not at the level of the final product, i.e., the protein. Indeed, changes in post-transcriptional and/or translational regulation mechanisms may lead to divergent observations between those two levels. In fact, this has already been observed in the context of 5-HT$_7$R. Thus, in 2007, Duncan and Franklin have not shown any age-induced modification of 5-HT$_7$R mRNA expression in the dorsal raphe nuclei (Duncan and Franklin, 2007). However, the same group of researchers observed a marked decrease of 50% at the protein level of 5-HT$_7$R in dorsal raphe nuclei in a prior study (Duncan et al., 1999). Thus, changes in the level of transcription of the 5-HT$_7$R mRNA did not account for the age-related difference observed at the protein level, at least in this brain structure. Besides, 5-HT$_7$R might also be putatively subjected, across aging, to modifications in their affinity or to changes in their coupling to G-proteins or other signaling pathways. In fact, such an effect has already been suggested by Duncan et al. (2004). Thus, even though a lack of data prevents us from drawing strong conclusions about the role of 5-HT$_7$R in the shift from spatial strategies across aging, those receptors appear to be promising target given their known locations in cerebral structures affected by aging processes, and their demonstrated role in spatial paradigms requiring the use of an allocentric strategy.

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The serotonin receptor 7 and the structural plasticity of brain circuits

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Serotonin (5-hydroxytryptamine, 5-HT) modulates numerous physiological processes in the nervous system. Together with its function as neurotransmitter, 5-HT regulates neurite outgrowth, dendritic spine shape and density, growth cone motility and synapse formation during development. In the mammalian brain 5-HT innervation is virtually ubiquitous and the diversity and specificity of its signaling and function arise from at least 20 different receptors, grouped in 7 classes. Here we will focus on the role 5-HT7 receptor (5-HT7R) in the correct establishment of neuronal cytoarchitecture during development, as also suggested by its involvement in several neurodevelopmental disorders. The emerging picture shows that this receptor is a key player contributing not only to shape brain networks during development but also to remodel neuronal wiring in the mature brain, thus controlling cognitive and emotional responses. The activation of 5-HT7R might be one of the mechanisms underlying the ability of the CNS to respond to different stimuli by modulation of its circuit configuration.

Keywords: 5-HT7R, brain connectivity, brain development, neurodevelopmental diseases, neuronal cytoarchitecture, serotonin

SEROTONIN AND BRAIN DEVELOPMENT

Serotonin is a neurotransmitter modulating numerous physiological processes in the nervous system such as sleep, mood, aggressive behavior, sexual behavior, sensory processing, cognitive control, emotion regulation, autonomic responses, and motor activity (for reviews see Daubert and Condron, 2010; Lesch and Waider, 2012). 5-HT was first discovered in the gut and enterochromaffin cells by the pharmacologist V. Erspamer in the middle thirties and subsequently in blood serum as a vasoconstrictor, hence the name serotonin (serum that gives tone; Rapport et al., 1948).

Serotonergic neurons are found in a variety of organisms, from C. Elegans to vertebrates. In mammals, they are among the earliest neurons being differentiated during development, and comprise a widely distributed neuronal network in the brain (Lesch and Waider, 2012).

Several experimental data have indicated that 5-HT may act as a signaling cue in the fetal brain during critical periods of development. It is recognized that 5-HT is synthesized early in embryonic development and its receptors are early expressed. In addition to the endogenous 5-HT, the brain of the fetus receives it also from the placenta of the mother, further emphasizing the importance of 5-HT in the early embryonic development of the brain. The contribution of these maternal-placental-fetal interactions appears to be critical for brain circuit wiring and for long-term brain functions (Bonnin et al., 2011). In particular, the 5-HT system plays a crucial role in the establishment of cortical circuits by controlling key cellular processes including neuronal migration and dendritic differentiation (Puig and Gulledge, 2011; Vitalis et al., 2013; Dayer, 2014). Cortical circuits control cognitive processes and their function is highly dependent on their structure that is shaped during development. Along this line, alteration of the 5-HT signaling system is associated to neurodevelopmental disorders affecting cognitive abilities, as mentioned below. Studies using genetic mouse models reveal that excessive 5-HT levels in the brain alter the correct development of mouse somatosensory cortex (Cases et al., 1996; Persico et al., 2001; Dayer, 2014). On the other hand, the depletion of 5-HT in the brain leads to behavioral and functional deficits, despite the lack of detectable cellular or morphological alterations in the CNS (Hendricks et al., 2003; Savelieva et al., 2008; Alenina et al., 2009). The absence of CNS evident morphological defects in these mouse models suggests that the lack of brain 5-HT may only affect fine shaping of specific circuits, so that these alterations are not revealed by gross morphological analyses of brain. On the other hand, transgenic mice with a 75% reduction in brain 5-HT levels showed reduced brain growth and delayed cortical maturation during postnatal life (Narboux-Nême et al., 2013). Interestingly, in a recent elegant experiment it was demonstrated that lack of brain 5-HT produced a striking reduction of serotonergic innervation in diencephalic areas (the suprachiasmatic and thalamic paraventricular nuclei) and a marked serotonergic hyperinnervation in forebrain areas (nucleus accumbens and hippocampus; Migliarini et al., 2013). These data strongly suggest that 5-HT can either promote or

inhibit terminal arborization of serotonergic axons depending on specific targets in the brain. In addition, these findings confirm that alterations of 5-HT levels during CNS development produce severe abnormalities in the serotonergic circuitry affecting the proper wiring of the brain. Consistently, numerous studies from both vertebrate and invertebrate organisms support the idea that 5-HT regulates neurite outgrowth and establishment of neuronal connectivity during brain development (Daubert and Condron, 2010; Lesch and Waider, 2012), and that alterations in early serotonin signaling may produce long-lasting changes. The latter might be the basis of a number of neuropsychiatric disorders which likely have developmental origins, such as schizophrenia, depression, affective disorders, anxiety and autism (Lesch and Waider, 2012; Velasquez et al., 2013; Dayer, 2014). Nevertheless, the key molecular and cellular events through which the 5-HT signaling affects brain connectivity are still poorly investigated. The high number of the 5-HT receptors and the lack of selective pharmacological agonists or inhibitors for the various subtypes have hampered a detailed analysis on their selective involvement in shaping brain networks and modulating neuronal cytoarchitecture. Actually, as mentioned, in the mammalian brain serotonergic neurons exert their effects through 20 subtypes of receptors that are grouped in 7 distinct classes based on pharmacological properties, amino acid sequences, gene organization, and their coupled second messenger pathways. All 5-HT receptors, with the exception of the 5-HT3, are typical G-protein-coupled-receptors (GPCRs) with seven transmembrane domains. The 5-HT3 receptor, instead, is a ligand-gated ion channel. This great diversity of receptors indicates the wide physiological role of 5-HT in the nervous system, whose complexity still needs to be elucidated (Pytliak et al., 2011; Gellynck et al., 2013).

THE SEROTONIN RECEPTOR 7

The 5-HT7 receptor was cloned independently by three laboratories in 1993 (Bard et al., 1993; Lovenberg et al., 1993; Ruat et al., 1993). A number of functional splice variants of this receptor have been identified due to the presence of introns in the 5-HT7R gene (Gellynck et al., 2013).

The 5-HT7R activates $G\alpha_s$ that stimulates adenylate cyclase, resulting in an increase in cAMP. The cAMP activates protein kinase A (PKA) leading to phosphorylation of different proteins (Leopoldo et al., 2011). The 5-HT7R is also coupled to stimulation of the mitogen-activated protein kinase extracellular signal-regulated kinases (ERK; Errico et al., 2001). More recently, the 5-HT7R has also been shown to interact with another member of the G protein family, the $G\alpha_{12}$. Activation of the 5-HT7R/$G\alpha_{12}$ signaling pathway leads to stimulation of Rho GTPases, Cdc42 and RhoA (Kvachnina et al., 2005; **Figure 1**).

The 5-HT7R is expressed in both the CNS and in peripheral tissues. In the CNS the receptor is expressed in the diencephalon, forebrain and in the Purkinje neurons of the cerebellum (for reviews see Matthys et al., 2011; Gellynck et al., 2013). The wide distribution of 5-HT7R in the brain reflects the numerous functions in which this receptor is implicated, such as circadian rhythms, sleep-wake cycle, thermoregulation (Leopoldo et al., 2011; Adriani et al., 2012; Monti et al., 2014; Romano et al., 2014) and nociception (Garcia et al., 2011), but also cognitive functions such as learning and memory processing (Roberts and Hedlund, 2012; Freret et al., 2014; Meneses, 2014). Importantly, the putative involvement of 5-HT7R in many neuropathological processes such as anxiety, schizophrenia, epilepsy, migraine, impulsivity and depression, cognitive and mood disturbances (Hedlund, 2009; Cates et al., 2013; Gellynck et al., 2013), makes it a potential target for new therapeutic applications.

To explain the fact that a single receptor is involved in such a variety of physio-pathological processes of the CNS, the GPCR dimerization has been proposed as a possible key mechanism that introduces diversity in 5-HT7 receptor signaling (Matthys et al., 2011). Accordingly, in a recent study, it has been shown that heterodimerization of 5-HT7 and 5-HT1A receptors differentially

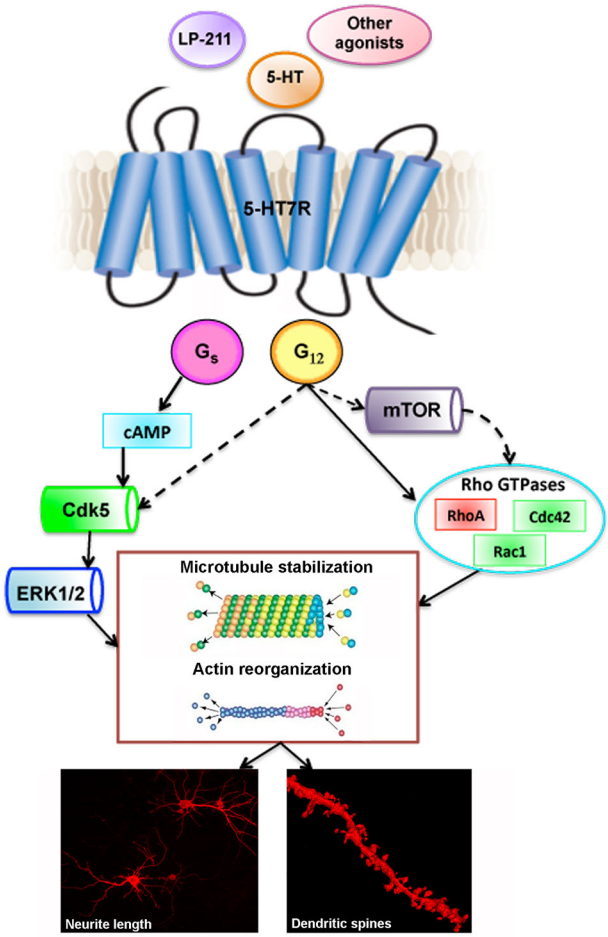

FIGURE 1 | Stimulation of 5-HT7R: schematic drawing of signaling pathways and downstream effectors leading to remodeling of neuronal morphology. Full lines indicate established pathways, dashed lines indicate putative targets. Lower panels are photomicrographs from DiI-labeled striatal neurons in culture (left panel), and striatal slices from brain adult mice (right panel, 3D- reconstruction). 5-HT: 5-hydroxytryptamine; 5-HT7R: serotonin receptor 7; LP-211: selective 5-HT7R agonist; Cdk5: cyclin-dependent kinase 5; ERK 1/2: extracellular signal-regulated kinases 1/2; mTOR: mammalian target of rapamycin.

regulates these receptors signaling and trafficking (Renner et al., 2012).

THE SEROTONIN RECEPTOR 7 SHAPES NEURONAL CIRCUITS DURING DEVELOPMENT

Numerous recent data indicate that the activation of 5-HT7R modulates neuronal morphology, excitability and plasticity contributing to the establishment of brain connectivity during embryonic and early postnatal life.

Using fibroblast cell lines, it was demonstrated that serotonin stimulation of 5-HT7R induces filopodia formation and cell rounding through interaction of the receptor with $G\alpha_{12}$ subunit of heterotrimeric G-protein and activation of Rho GTPases. This finding prompted more detailed studies using mouse hippocampal neurons in culture, where activation of the endogenous 5-HT7R, using the agonist 5-CT, determines pronounced extension of neurite length (Kvachnina et al., 2005). These results have been confirmed by more recent analyses where 5-HT7R was stimulated with 5-HT, or with the selective agonist AS19, or with the 5-$HT_{1A/7}$ receptor agonist 8-OH-DPAT (Tajiri et al., 2012; Rojas et al., 2014). The effects of the 5-CT, as well as those of the 8-OH-DPAT were abolished by the co-treatment with 5-HT7R selective antagonist SB-269970, indicating the specific involvement of 5-HT7R. The morphogenic effects of the 5-HT7R on neuronal cytoarchitecture have been demonstrated also for neurons from additional CNS areas. Treatment of cultured embryonic neurons from rodent striatal complex and cortex with 8-OH-DPAT and with the highly potent and selective 5-HT7R agonist LP-211, significantly enhances neurite outgrowth through pathways involving Cdk5 and extracellular signal-regulated kinases 1/2 (ERK). These effects are selectively due to the 5-HT7R stimulation since they are blocked by SB-269970. Neurite elongation requires de novo protein synthesis and is accompanied by qualitative and quantitative modifications of selected cytoskeletal proteins (Speranza et al., 2013). These findings delineate an overall picture of potential intracellular pathways and molecular mechanisms that underlie modulation of neuronal morphology due to 5-HT7R stimulation (**Figure 1**).

In accordance with the morphogenic role of 5-HT7R, it was demonstrated that prolonged stimulation of the 5-HT7R/$G\alpha_{12}$ signaling pathway in early postnatal cultured hippocampal neurons leads to an increased number of dendritic protrusions and synaptic contacts, and enhances spontaneous synaptic activity. A similar morphogenic function of the 5-HT7R was confirmed in organized brain circuitries (organotypic slices preparation from the hippocampus of juvenile mice), where stimulation of 5-HT7R/$G\alpha_{12}$ signaling pathway potentiates formation of dendritic spines, increases neuronal excitability and modulates synaptic plasticity (LTP). The latter effect was age-dependent, indeed it was observed in 1 week-old mice but not in adult animals, probably due to decreased hippocampal expression of the 5-HT7R during later post-natal stages (Kobe et al., 2012).

Intriguingly, 5-HT7R modulates NMDA receptors activity in hippocampal neurons. Long term activation of the 5-HT7R by the selective agonist LP 12 inhibits glutamate receptor signaling preventing NMDA-induced neurotoxicity (Vasefi et al., 2013a), while acute activation of 5-HT7R promotes NMDA receptor activity (Vasefi et al., 2013b).

In addition, activation of 5-HT7R modulates long-term depression mediated by metabotropic glutamate receptors in wild-type as well as in a mouse model of Fragile X- syndrome (FXS; Costa et al., 2012). These animals exhibit spatial memory impairment and synapse malfunctioning in the hippocampus, with abnormal enhancement of mGluR-LTD. Abnormal LTD might lead to excessive synapse elimination, whereas physiological LTD is crucial in hippocampal-dependent memory. The activation of 5-HT7R by 5-HT, or 8-OH-DPAT, or LP-211 in hippocampal slices from the FXS mouse model was able to correct excessive mGluR-LTD, bringing it back to its physiological level and thereby restoring synaptic plasticity (Costa et al., 2012). Hippocampal LTP and LTD are the most studied paradigms of synaptic plasticity that cause enduring strengthening and weakening of synapses, paralleled by increase and decrease of dendritic spine volume (Bosch and Hayashi, 2012). These data indicate that brain plasticity is accompanied by modification of neuronal connectivity and formation of new neuronal circuits. The molecular and cellular mechanisms of this modulation are still only partially known, but 5-HT7R seems a good candidate to be involved in the molecular cascade.

THE SEROTONIN RECEPTOR 7 MODULATES CONNECTIVITY IN ADOLESCENT AND MATURE BRAIN

In addition to the involvement of 5-HT7R in neuronal cytoarchitecture and network construction during embryonic and early postnatal life, 5-HTR7 seems to play a role in the modulation of structural plasticity in adolescent and mature brain circuits. Indeed it is now widely accepted that mature mammalian brain undergoes dramatic structural reorganization with time and experience (Holtmaat and Svoboda, 2009; Sala and Segal, 2014). Intriguingly, it has been demonstrated that brain wiring may be modulated by chronic pharmacological intervention, as indicated by the comprehensive phenotype correction, including dendritic spine density, in adult mice models of FXS treated in young adulthood with a selective mGlu5 inhibitor (Michalon et al., 2012).

In adolescent rodents, it has been hypothesized that 5-HT7R may subserve the persistent structural rearrangements of the brain reward pathways occurring during postnatal development, following chronic methylphenidate exposure (Adriani et al., 2006; Leo et al., 2009). Accordingly, stimulation of 5-HT7R in adolescent rats by intraperitoneal administration of LP-211 (0.25 mg/kg/day for 5 days), induces plastic rearrangements within forebrain networks, accounting for long-lasting behavioral changes in the adulthood (Canese et al., 2014). Similar results were obtained in a rat model for Attention Deficit Hyperactivity Disorder (ADHD) in which prepuberal stimulation of 5-HT7R by intraperitoneal administration of LP-211 (up to 0.5 mg/kg/day for 14 days), has long-term effects on adult behavior, improving spatial attention and resulting in modified expression of pre- and post-synaptic markers (Ruocco et al., 2014a), while the same treatment, during adolescence, modulates the emotional responses (Ruocco et al., 2014b). In addition, stimulation of 5-HT7R exerted consistent effects into exploratory motivation,

anxiety-related profiles and spontaneous circadian rhythm in adult rodents (Adriani et al., 2012).

Most of these experiments have been performed using LP-211, a novel highly potent and selective 5-HTR7 agonist that, in being brain-penetrant, is particularly useful for *in vivo* studies (Hedlund et al., 2010). Thus, adult mice treated *in vivo* with intraperitoneal injection of LP-211 (0.25 mg/kg/day for 3 days) showed a significant increase in the total number and density of dendritic spines in neurons of the dorso-lateral striatum (Speranza et al., in preparation for this issue). In view of the fact that dendritic spines actively participate in the formation of synapses, these data strongly support the notion that this receptor may be involved in shaping the neuronal wiring of the mature CNS.

Along this line, LP-211 stimulation of 5-HT7R by intraperitoneal administration of LP-211 (0.25 mg/kg/day for 7 days) in an adult mouse model of Rett Syndrome (the MeCP2-308 strain) was able to rescue the behavioral deficits and to reverse the abnormal activation of the key molecules regulating actin cytoskeleton dynamics, which in turn modulate neuronal morphology (De Filippis et al., 2014). In addition, inhibition of 5-HT7R with the selective antagonist SB-269970 was able to ameliorate psychomotor and cognitive deficits in animal model of schizophrenia (PACAP-deficient mice), supporting the notion that 5-HT7R is linked to the already mentioned psychiatric disorders such as schizophrenia and depression (Tajiri et al., 2012). This view has been further supported by independent experiments using lurasidone, a novel atypical antipsychotic drug with a powerful antagonist activity for 5-HT7R. Lurasidone ameliorates learning and memory deficits in animal model of schizophrenia and induces an antidepressant-like response in animal models for depression and anxiety. Interestingly, these pharmacological actions of lurasidone are mediated, at least partially, by 5-HT7R (Ishibashi et al., 2010; Cates et al., 2013; Horisawa et al., 2013), corroborating previous data that demonstrate the involvement of 5-HT7R in depression (Hedlund et al., 2005; Mnie-Filali et al., 2007).

The 5-HT7R expression in brain regions involved in learning and memory parallels with its functions. The 5-HT7R knockout mice exhibits specific impairments in contextual learning (Roberts et al., 2004). Several other studies highlight the implication of 5-HT7R in memory and attention-related processes (Gasbarri et al., 2008; Freret et al., 2014; Meneses, 2014), underscoring its role in the modulation of the neuronal network associated with cognitive functions. Therefore, the study of this receptor and its associated intracellular pathways may provide invaluable information for the treatment of learning and memory disorders. From a general point of view, the involvement of 5-HT7R in such numerous neurological disorders associated with abnormal CNS connectivity, recognizes this receptor as a promising target for the development of innovative therapeutical strategies.

CONCLUSION

Taken together the results highlighted here indicate that 5-HT7R is an important player involved in the establishment of neuronal cytoarchitecture during development of CNS, and strongly suggest its modulatory action in remodeling neuronal morphology and circuitry in the mature brain. Future studies using high resolution *in vivo* imaging, coupled with the elucidation of molecular mechanisms responsible for morphological modifications will further our knowledge on 5-HT7R role in brain plasticity.

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Novel agonists for serotonin 5-HT$_7$ receptors reverse metabotropic glutamate receptor-mediated long-term depression in the hippocampus of wild-type and Fmr1 KO mice, a model of Fragile X Syndrome

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Serotonin 5-HT$_7$ receptors are expressed in the hippocampus and modulate the excitability of hippocampal neurons. We have previously shown that 5-HT$_7$ receptors modulate glutamate-mediated hippocampal synaptic transmission and long-term synaptic plasticity. In particular, we have shown that activation of 5-HT$_7$ receptors reversed metabotropic glutamate receptor-mediated long-term depression (mGluR-LTD) in wild-type (wt) and in Fmr1 KO mice, a mouse model of Fragile X Syndrome in which mGluR-LTD is abnormally enhanced, suggesting that 5-HT$_7$ receptor agonists might be envisaged as a novel therapeutic strategy for Fragile X Syndrome. In this perspective, we have characterized the basic *in vitro* pharmacokinetic properties of novel molecules with high binding affinity and selectivity for 5-HT$_7$ receptors and we have tested their effects on synaptic plasticity using patch clamp on acute hippocampal slices. Here we show that LP-211, a high affinity selective agonist of 5-HT$_7$ receptors, reverses mGluR-LTD in wt and Fmr1 KO mice, correcting a synaptic malfunction in the mouse model of Fragile X Syndrome. Among novel putative agonists of 5-HT$_7$ receptors, the compound BA-10 displayed improved affinity and selectivity for 5-HT$_7$ receptors and improved *in vitro* pharmacokinetic properties with respect to LP-211. BA-10 significantly reversed mGluR-LTD in the CA3-CA1 synapse in wt and Fmr1KO mice, indicating that BA-10 behaved as a highly effective agonist of 5-HT$_7$ receptors and reduced exaggerated mGluR-LTD in a mouse model of Fragile X Syndrome. On the other side, the compounds RA-7 and PM-20, respectively arising from *in vivo* metabolism of LP-211 and BA-10, had no effect on mGluR-LTD thus did not behave as agonists of 5-HT$_7$ receptors in our conditions. The present results provide information about the structure-activity relationship of novel 5-HT$_7$ receptor agonists and indicate that LP-211 and BA-10 might be used as novel pharmacological tools for the therapy of Fragile X Syndrome.

Keywords: 5-HT7, 5-HT7R agonists, hippocampus, mGluR-LTD, Fragile X Syndrome

Introduction

Serotonin (5-HT) is a monoamine neurotransmitter controlling several physiological functions among which mood, circadian rhythms, body temperature, food intake and nociception. Seven main families (5-HT_1 through 5-HT_7) and at least 14 subtypes of 5-HT receptors have been identified to date (Hannon and Hoyer, 2008; Nichols and Nichols, 2008). In the last decade, an important role of 5-HT_7 receptors in learning and memory has emerged. Serotonin is released in the hippocampus (one of the brain structures most crucially involved in learning) by fibers arising from raphe nuclei (Segal, 1990; Schmitz et al., 1998). 5-HT_7 receptors are expressed in the hippocampus and modulate neuronal excitability and synaptic transmission (Costa et al., 2012b). Behavioral studies performed on wild-type animals (Perez-Garcia and Meneses, 2005; Eriksson et al., 2008; Freret et al., 2014; Meneses et al., 2015) and on mice lacking 5-HT_7 receptors (5-HT_7 KO) (Sarkisyan and Hedlund, 2009) indicate that 5-HT_7 receptor activation exerts a pro-cognitive action. In light of this, 5-HT_7 receptors have recently been proposed as a novel target in cognitive diseases (Matthys et al., 2011; Ciranna and Catania, 2014; Gasbarri and Pompili, 2014).

Fragile X Syndrome (FXS) is the most common form of inherited intellectual disability, frequently associated with mood disorders (Hagerman and Hagerman, 2002; Garber et al., 2008), autism (Harris et al., 2008) and increased susceptibility to seizures (Musumeci et al., 2001). FXS is caused by silencing of the Fmr1 gene coding for Fragile X Mental Retardation Protein (FMRP) (Pieretti et al., 1991), an mRNA binding protein that functions as a regulator of protein translation (Laggerbauer et al., 2001; Bechara et al., 2009). One of the main consequences of FMRP absence is a dysregulation of protein synthesis, leading to altered synapse morphology and synaptic dysfunction (Bhakar et al., 2012). The brain of FXS patients shows abnormal dendrite morphology in cortex and hippocampus, with an overproduction of long, thin and immature dendritic spines (Irwin et al., 2000). Evidence of synapse malfunction came from studies on the Fmr1 gene knockout (Fmr1 KO) mouse, an animal model of FXS that displays typical features resembling those of FXS patients, among which alterations in dendritic spine morphology (Comery et al., 1997; Nimchinsky et al., 2001) increased susceptibility to audiogenic seizures (Musumeci et al., 2000) and cognitive impairment (Bernardet and Crusio, 2006; Dolen et al., 2007). *In vitro* studies on Fmr1 KO mice revealed that the lack of FMRP dysregulates protein translation induced by activation of group I metabotropic glutamate receptors (mGluRs), with an overproduction of proteins involved in AMPA receptor endocytosis (Nakamoto et al., 2007). Consistently, mGluR-mediated long-term depression (mGluR-LTD), a form of LTD mediated by activation of mGlu5 receptors and leading to endocytosis of AMPA receptors, is abnormally enhanced in the hippocampus of Fmr1 KO mice (Huber et al., 2002). In line with the "mGluR" theory of Fragile X Syndrome, pharmacological blockade or reduced expression of group I mGlu receptors were found to rescue cognitive impairment and abnormal behavior in Fmr1 KO mice (Luscher and Huber, 2010; Bhakar et al., 2012).

We have recently found that 5-HT_7 receptor activation is able to reverse mGluR-LTD and mGluR-mediated endocytosis of AMPA receptors both in wild-type and in Fmr1 KO mice (Costa et al., 2012a). These data suggest that abnormal mGluR-mediated signaling in Fmr1 KO mice can be reversed by serotonin acting through 5-HT_7 receptors, suggesting that selective 5-HT_7 receptor agonists might become novel pharmacological tools for FXS therapy.

In the present work, we have studied the effect of LP-211, a selective and brain-penetrant agonist of 5-HT_7 receptors (Leopoldo et al., 2008; Hedlund et al., 2010) and of novel putative agonists of 5-HT_7 receptors in view of future preclinical studies on Fmr1 KO mice.

Materials and Methods

Synthesis of 5-HT_7 Receptor Ligands

The synthesis of the following 5-HT_7 receptor ligands was accomplished as previously reported: *N*-(4-cyanophenylmethyl)-4-(2-diphenyl)-1-piperazinehexanamide, LP-211 (Leopoldo et al., 2008), (*R*)-1-[4-[2-(4-methoxyphenyl)phenyl]piperazin-1-yl]-3-(2-pyrazinyloxy)-2-propanol, BA-10 (Hansen et al., 2014), *N*-(2-fluoropyridin-5-ylmethyl)-4-[2-(4-methoxy)phenyl]-1-piperazinehexanamide, PF-62 (Lacivita et al., 2014), 1-(2-biphenyl)piperazine, RA-7 (Bantle, 1996), 1-[2-(4-methoxyphenyl)phenyl]piperazine, PM-20 (Lacivita et al., 2012). *N*-(4-cyanophenylmethyl)-3-[4-[2-(4-methoxyphenyl)phenyl]pipe-razin-1-yl]ethoxy]propanamide, MM-1, and *N*-(4-trifuoro methylphenylmethyl)-3-[4-[2-(4-methoxyphenyl)phenyl]piperazin-1-yl]ethoxy]propanamide, MM-2, were synthesized as depicted in **Figure 1** (Scheme 1). RA-7 was alkylated with 2-bromoethanol to give alcohol 1 that underwent Michael addition to t-butylacrylate to afford the ester 2. Acidic hydrolysis of the ester afforded carboxylic acid 3 that was subsequently condensed with 4-cyanophenylmethylamine or 4-trifuoromethylphenylmethylamine to give the target compounds MM-1 and MM-2, respectively. These synthetic procedures involved purification of intermediate and target compounds by column chromatography on silica gel column. The chemical structure of intermediate and target compounds was confirmed by NMR spectroscopy, mass spectrometry and elemental analysis.

Evaluation of Metabolic Stability of Novel High-Affinity 5-HT_7 Ligands

In vitro metabolism of compounds BA-10, PF-62, MM-2, and MM-1 was evaluated after incubation with mouse hepatic microsomes in the presence of an NADPH-generating system, according to Jia and Liu (Jia and Liu, 2007). Test compounds were pre-incubated at 37°C with mouse liver microsomes (0.5 mg/mL microsomal protein) at 1 μM final concentration in 100 mM potassium phosphate buffer (pH 7.4) for 10 min. Metabolic reactions were initiated by the addition of a NADPH regenerating system (NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, final glucose-6-phosphate dehydrogenase concentration, 1 unit/mL). Aliquots were removed (0, 5, 10, 30, and 60 min) and immediately mixed with an equal volume of cold acetonitrile

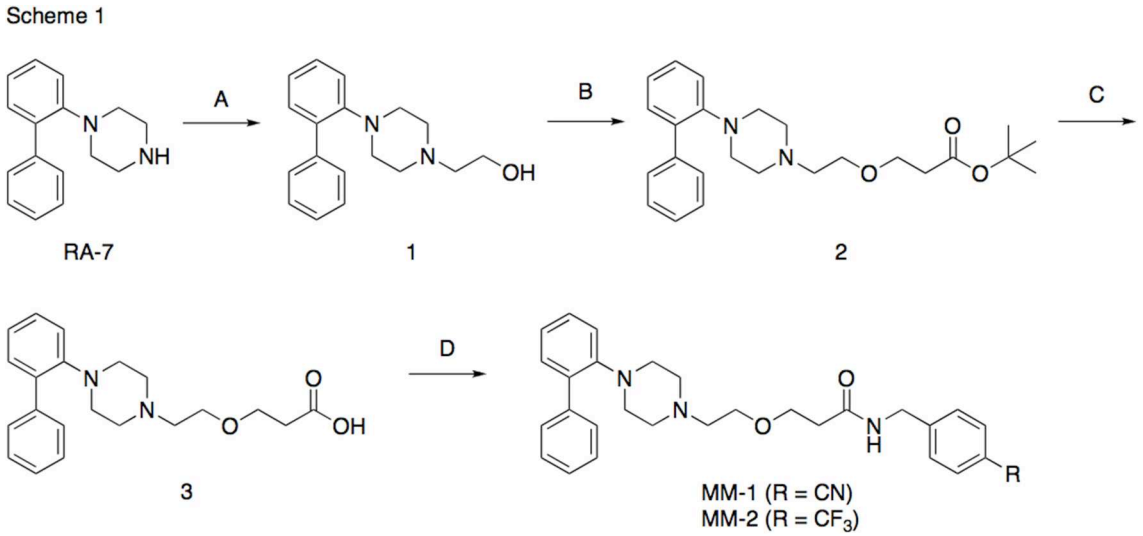


FIGURE 1 | Synthesis of Compounds MM-1 and MM-2. Reagents and Conditions: (A) 2-bromoethanol, K_2CO_3, KI, acetonitrile, reflux overnight, 82% yield; (B) NaH, *t*-butyl acrylate, anhydrous THF, 0°C to r.t., 20–22 h, 43% yield; (C) 3N HCl, dioxane, r.t., 48 h, quantitative yield; (D) 4-cyanophenylmethylamine or 4-trifuoromethylphenylmethylamine, 1,1'-carbonyldimidazole, anhydrous THF, r.t., overnight, 95% yield.

containing internal standard. Test compound incubated with microsomes without NADPH regenerating system for 60 min was included. Quenched samples were centrifugated at 4500 rpm for 15 min and the supernatants were analyzed by reversed phase HPLC. Quantitation of the parent compound at the various time points was performed using Agilent 1290 Infinity LC System equipped with diode array detector. The column used was a Phenomenex Gemini C-18 (250 × 4.6 mm, 5 μm particle size). The samples were isocratically eluted using CH_3CN/ammonium formate (20 mM, pH 5.5) 7:3 (v/v) as mobile phase. The chromatograms were registered at three different wavelenghts (230, 254, and 280 nm).

Natural logarithm of percentage remaining vs. time data for each compound was fitted to linear regression and the slope was used to calculate the degradation half-life ($t_{1/2}$) according to the equation (Obach et al., 1997):

$$t_{1/2} : \frac{0.693}{k}$$

In vitro half-life was then used to calculate the intrinsic plasma clearance (CL_{int}) according to the following equation (Obach et al., 1997):

$$CL_{int} \quad \frac{0.693}{t_{1/2}} \bullet \frac{\text{volume of incubation } (\mu L)}{\text{protein in the incubation}}$$

Electrophysiology

Patch clamp experiments were performed on mouse hippocampal slices from wild-type (wt) mice (FVB and C57BL/6J background) and Fmr1 KO2 mice (C57BL/6J background, kindly provided by Prof. Willemsen, Erasmus MC, Rotterdam, The Netherlands). Animal care and experimental procedures were performed in accordance with the European Communities Council guidelines 86/609/EEC. Every care was taken to maximally reduce the number of animals and to minimize discomfort.

Acute hippocampal slices from wt and Fmr1 KO mice (age 14–21 days) were prepared as previously described (Costa et al., 2012a,b). Briefly, the brain was rapidly removed and placed in oxygenated ice-cold artificial cerebro-spinal fluid (ACSF; in mM NaCl 124; KCl 3.0; NaH_2PO_4 1.2; $MgSO_4$ 1.2; $CaCl_2$ 2.0; $NaHCO_3$ 26; D-glucose 10, pH 7.3). Acute hippocampal slices (300 μM) were cut with a vibratome (Leica VT 1200). Slices were continually perfused with oxygenated ACSF. After 3 h of recovery, one slice was placed in the recording chamber of a patch camp set up and viewed with infrared microscopy (Leica DMLFS). A tungsten electrode was placed in the *stratum radiatum* to stimulate Schaffer collaterals with negative current pulses (duration 0.3 ms, delivered every 15 s by A310 Accupulser, WPI, USA). Evoked AMPA receptor-mediated excitatory post-synaptic currents (EPSCs) were recorded from CA1 pyramidal neurons under whole-cell patch clamp (holding potential -70 mV; EPC7-plus amplifier HEKA, Germany). Stimulation intensity was set to induce half-maximal EPSC amplitude. Series resistance (Rs) was monitored throughout the recording by means of 10 mV hyperpolarizing pulses; recordings were discarded from analysis if Rs changed by more than 20%. EPSC traces were filtered at 3 kHz and digitized at 10 kHz. Data were acquired and analyzed using Signal software (CED, England). The recording micropipette (resistance 1.5–3 MΩ) was filled with intracellular solution (in mM: K-gluconate 140; HEPES 10; NaCl 10; $MgCl_2$ 2; EGTA 0.2; Mg-ATP 3.5; Na-GTP 1; pH 7.3). Bath solution (ACSF; flow rate of 1.5 ml/min) routinely contained (-)-bicuculline methiodide (5 μM, SIGMA) and D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5, 50 μM, Tocris). mGluR-LTD of synaptic currents was chemically induced by application

of the group I mGluR agonist DHPG (100 μM, 5 min) in the absence and in the presence of a 5-HT_7 receptor ligand. Pharmacological agents (*S*-3,5-DHPG 100 μM; LP-211 10 nM; BA-10 10 nM; RA-7 10 nM; PM-20 10 nM) were dissolved in ACSF and applied by bath perfusion.

For LTD data analysis, peak amplitude values of evoked EPSCs were averaged over 1 min and expressed as % of control (calculated from EPSCs recorded during at least 15 min before DHPG application). The amount of mGluR-LTD was calculated 45–55 min after LTD induction by DHPG application and is expressed by indicating EPSC amplitude as percentage of baseline EPSC (% EPSC). Cumulative histograms indicate % EPSC amplitude (mean ± SEM from groups of neurons) after application of DHPG alone (control LTD) or DHPG followed by a 5-HT_7 receptor agonist. EPSC amplitude values from two groups of neurons were compared using the Student's *t*-test, with *n* indicating the number of neurons tested in each condition.

Results

The 5-HT_7 Receptor Agonist LP-211 reversed mGluR-LTD in Wild-Type and Fmr1 KO Mice

Excitatory post-synaptic currents (EPSCs) mediated by glutamate AMPA receptors were recorded from CA1 pyramidal neurons following stimulation of Schaffer collaterals in hippocampal slices from wt and Fmr1 KO mice (C57BL6J background; post natal day PN 14–21).

In slices from wt mice, bath application of DHPG (100 μM, 5 min), an agonist of group I metabotropic glutamate receptors, induced a long term depression (mGluR-LTD) of synaptic currents (**Figure 2A**): EPSC amplitude measured 45 min after DHPG application (74.4 ± 9.8, mean ± SEM, $n = 9$) was significantly lower than control EPSC amplitude (99.97 ± 3.2, $n = 9$; $P < 0.01$; **Figure 2B**).

In another group of recordings, LP-211 (10 nM, 5 min) was applied 5 min after DHPG (**Figure 2A**): in these conditions, EPSC amplitude measured 45 min after DHPG application (99.4 ± 1.5% of control, mean ± SEM, $n = 5$) was not significantly different from control EPSC amplitude ($P = 0.7$, **Figure 2B**), indicating that mGluR-LTD was completely reversed by LP-211. This result fully confirms our previous data obtained using the FVB mouse strain, showing that LP-211 reversed mGluR-LTD in hippocampal slices from wild-type mice (Costa et al., 2012a).

In slices from Fmr1 KO mice (C57BL6J background), application of DHPG strongly reduced EPSC amplitude (EPSC measured 45 min after DHPG: 56 ± 5% of control, mean ± SEM, $n = 7$; **Figure 2C**), with a highly significant reduction with respect to control EPSC ($P < 0.00001$, **Figure 2D**). The amount of DHPG-induced inhibition in slices from Fmr1 KO mice was significantly larger than in wt slices (EPSC amplitude: 74 ± 4% of control, mean ± SEM, $n = 9$; $P = 0.0069$; compare black columns in **Figures 2B,D**). This result confirms that mGluR-LTD in Fmr1 KO mice was enhanced with respect to wt, in agreement with previous data (Huber et al., 2002; Zhang et al., 2009; Choi et al., 2011; Costa et al., 2012a).

Application of LP-211 after DHPG reversed mGluR-LTD also in slices from Fmr1 KO mice (**Figure 2C**). Compared to wt, reversal of mGluR-LTD by LP-211 in Fmr1 KO slices had a slower time course: 45 min after DHPG application, the amount of mGluR-LTD was still significant (EPSC amplitude 78 ± 5% of control, mean ± SEM, $n = 6$, $P = 0.001$, **Figure 2D**, upper panel), but was significantly lower ($P < 0.01$) than that induced by DHPG only. A full reversal of mGluR-LTD by LP-211 was observed 55 min after DHPG application: EPSC amplitude (97 ± 3% of control, mean ± SEM, $n = 6$) was not significantly different from control EPSC ($P = 0.07$; **Figure 2D**, lower panel).

In Vitro Pharmacokinetic Properties of New Putative 5-HT_7 Receptor Agonists

The four new putative 5-HT_7 receptor agonists BA-10, PF-62, MM-2, and MM-1 were identified in our laboratory following a medicinal chemistry campaign to obtain 5-HT_7 receptor ligands with the same structural scaffold of LP-211 but endowed with improved metabolic stability (**Table 1**). These molecules incorporated chemical features known to improve metabolic stability, such as electron withdrawing groups, or polar groups that reduce the lipophilicity of the molecule, since it is known that high lipophilicity is correlated with metabolic liability (Mannhold, 2006). The new compounds were initially tested for their binding affinity for 5-HT_7 receptors. With respect to LP-211, BA-10, and PF-62 displayed an increased affinity for 5-HT_7 receptors, whereas MM-1 and MM-2 respectively showed comparable and lower affinity (**Table 1**). This indicated the correct design of the new molecule that was aimed to obtain compounds with the same profile as LP-211. Interestingly, all the compounds tested showed improved selectivity for 5-HT_7 over 5-HT_{1A} receptors with respect to LP-211 (**Table 1**).

In vitro metabolism of compounds BA-10, PF-62, MM-2, and MM-1 was evaluated after incubation with mouse hepatic microsomes in the presence of an NADPH-generating system, according to Jia and Liu (2007). Liver microsomes provide the most convenient way to study cytochrome P450 enzyme-mediated metabolism. Half-life ($t_{1/2}$) and Intrinsic Clearance (CL_{Int}) of the test compounds are listed in **Table 1**. Half-life and intrinsic clearance of MM-2 and MM-1 indicated that these compounds are less metabolically stable than PF-62, LP-211 and BA-10. Instead, BA-10 appeared endowed with higher metabolic stability than LP-211. In particular, BA-10 displayed the longest *in vitro* half-life among the five compounds examined, indicating improved metabolic stability with respect to LP-211.

We next evaluated the efflux ratio (BA/AB) of PF-62, MM-2, and MM-1 between basal-to-apical (BA) and apical-to-basal (AB) fluxes in Caco-2 cells monolayer. This system is an *in vitro* model to evaluate the interaction of small molecules with P-glycoprotein (P-gp), an efflux pump localized on the blood-brain barrier that prevents xenobiotics to enter the brain. Therefore, a centrally acting drug should not be a P-gp substrate. In this system, a cutoff value of 3 is generally used to distinguish P-gp substrate from nonsubstrate (Hitchcock, 2012). BA/AB ratios of the test compounds are listed in **Table 1**: all the compounds tested appeared to be weak substrates, thus should only weakly interact with P-gp.

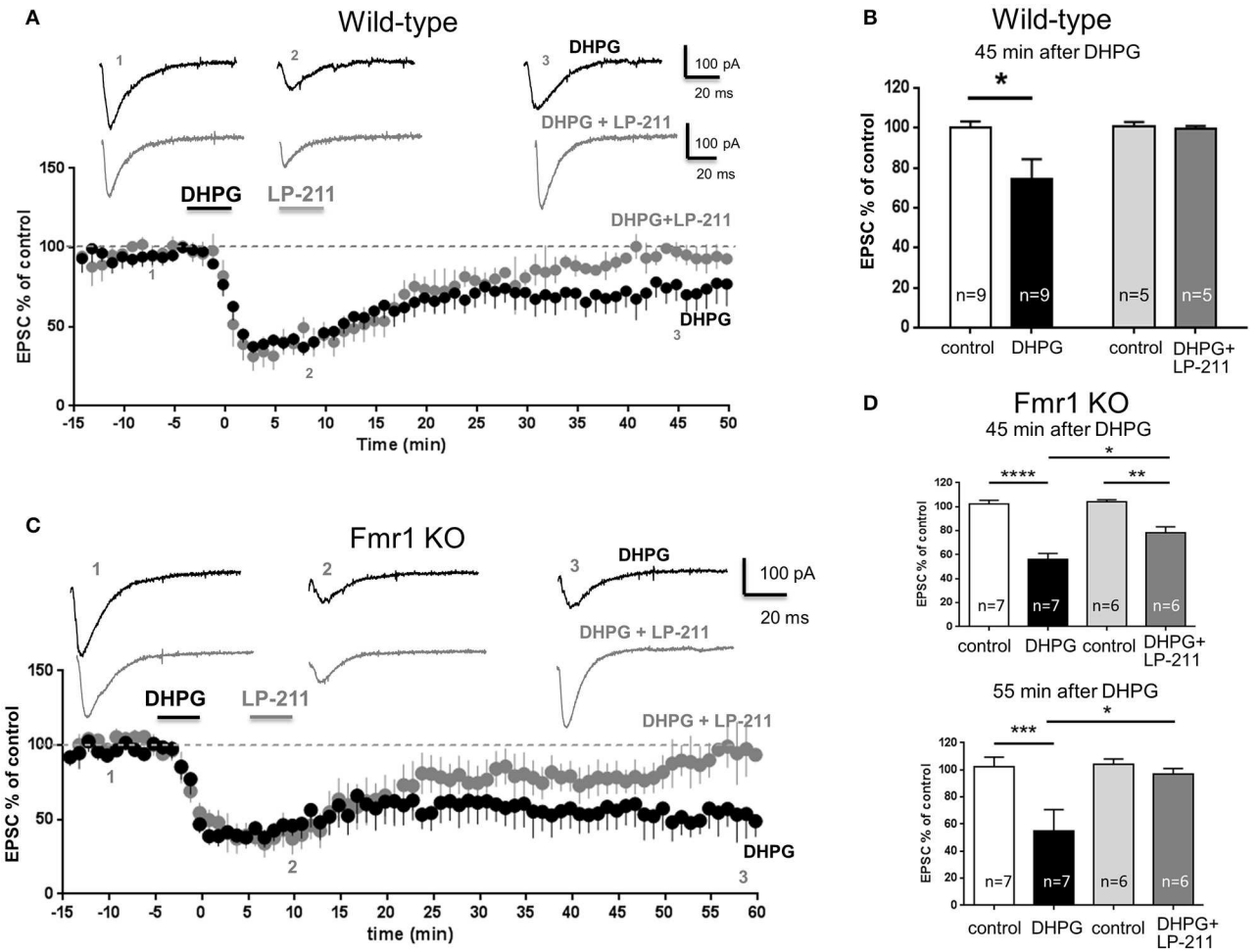
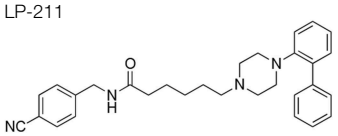
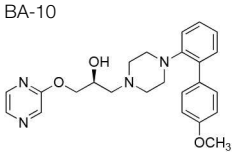
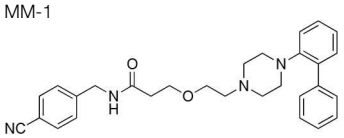
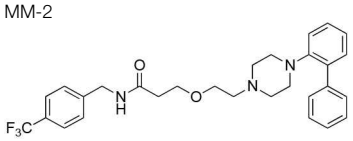
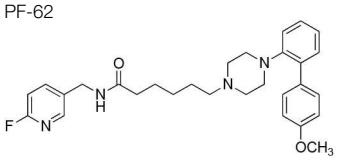


FIGURE 2 | LP-211 reversed mGluR-LTD in the hippocampus of wild-type and Fmr1 KO mice. Excitatory post-synaptic currents (EPSCs) mediated by AMPA receptors were recorded under whole-cell patch clamp from CA1 pyramidal neurons after stimulation of Schaffer collaterals in the continuous presence of the NMDA receptor antagonist D-AP5 (50 μM). EPSC amplitude is represented as percent of control EPSC amplitude prior to any substance application. Pooled data of % EPSC values (mean ± SEM) were plotted vs. time; insets show individual EPSC traces from representative experiments. **(A)** Hippocampal slices (300 μm) were prepared from wild-type mice on a C57BL/6J background (PN 14-21). Bath application of DHPG (100 μM, 5 min), an agonist of group I metabotropic glutamate receptors, induced a long term depression of EPSC amplitude. In another group of recordings, LP-211 (10 nM, 5 min) was applied 5 min after LTD induction. Following application of LP-211, EPSC amplitude returned to control values (100%), indicating that mGluR-LTD was reversed. **(B)** Histograms show mean EPSC amplitude values (% of control) in control conditions (empty column, $n = 9$) and 45 min after application of DHPG alone (black column, $n = 9$) or application of DHPG followed by LP-211 (dark gray column, $n = 5$). Application of DHPG significantly reduced EPSC amplitude ($^*P < 0.01$), thus induced mGluR-LTD. Vice-versa, when DHPG application was followed by LP-211, EPSC values were not reduced with respect to control, indicating that mGluR-LTD was completely reversed. **(C)** Hippocampal slices were prepared from Fmr1 KO mice (C57BL6J strain; PN 14-21) Application of DHPG (100 μM, 5 min) induced a long-term depression (mGluR-LTD) of EPSC amplitude. When LP-211 (10 nM, 5 min) was applied after DHPG, mGluR-LTD was partially reversed. A complete reversal of mGluR-LTD was observed around 55 min after LTD induction. **(D),** upper panel: EPSC current amplitude in control conditions (100%) and 45 min after application of DHPG. In slices from Fmr1 KO mice, the amount of mGluR-LTD induced by DHPG alone was highly significant (black column; $^{****}P < 0.00001$) and was larger than in wild-type (compare with panel **B**). When DHPG application was followed by application of LP-211, the amount of mGluR-LTD was still significant ($^{**}P < 0.001$) but was significantly reduced ($^*P < 0.01$) with respect to mGluR-LTD induced by DHPG alone, indicating that mGluR-LTD was partially reversed. **(D)** Lower panel: EPSC current amplitude in control conditions (100%) and 55 min after application of DHPG: mGluR-LTD was completely reversed by LP-211, indicating a slower time course of LP-211-mediated reversal in Fmr1 KO with respect to wild-type.

In line with this, a recent positron emission tomography study performed with [^{11}C]BA-10 clearly showed *in vivo* that BA-10 is not a P-gp substrate, being able to massively accumulate into the brain (Hansen et al., 2014), thus is likely to act on the central nervous system following systemic administration.

In summary, among the new putative 5-HT$_7$ receptor agonists examined in the present study, the compound BA-10 displayed improved affinity and selectivity for 5-HT$_7$ receptors, together with improved *in vitro* metabolic stability with respect to the 5-HT$_7$ receptor agonist LP-211.

TABLE 1 | Binding Affinity Data and Pharmacokinetic Parameters of Test Compounds.

Compound	5-HT$_7$ K_i [nM]	5-HT$_{1A}$ K_i [nM]	$t_{1/2}$ (min)	CL_{int} (μL/mg/min)	BA/AB
LP-211 	15	188	10.6	131	4.0
BA-10 	1.1	242	14.8	96	5.6
MM-1 	16	437	2.7	515	3.3
MM-2 	35	582	2.8	498	3.5
PF-62 	3.82	152	6.7	205	4.2

The Novel Compound BA-10 Behaved as an Agonist of 5-HT$_7$ Receptors and Reversed mGluR-LTD in the Hippocampus of Wild-Type and Fmr1 KO Mice

We next tested the biological effect of BA-10 on native 5-HT$_7$ receptors in the hippocampus of wild-type mice (PN 14–21). When BA-10 (10 nM, 5 min) was applied after DHPG, EPSC amplitude returned to values comparable to control (EPSC amplitude 45 min after DHPG application: 107 ± 23% of control, mean ± SEM, $n = 6$; **Figures 3A,B**), indicating that mGluR-LTD was reversed.

In slices from Fmr1KO mice (PN 14–20), application of BA-10 completely reversed DHPG-induced mGluR-LTD (**Figure 3C**): DHPG induced a significant LTD when applied alone ($P = 0.01$, **Figure 3D**) but not when applied together with BA-10 ($P = 0.29$, **Figure 3D**). The amount of mGluR-LTD after application of DHPG alone or DHPG with BA-10 was significantly different (EPSC amplitude respectively 56 ± 12% and 116 ± 11% of control; $P = 0.0054$; **Figure 3D**).

These results indicate that BA-10, a novel compound with improved pharmacokinetic properties with respect to known 5-HT$_7$ receptor agonists, is able to reverse mGluR-LTD in wild-type mice and to rescue synaptic plasticity in Fmr1 KO mice, thus might be envisaged as a new pharmacological tool for Fragile X Syndrome.

The Compounds RA-7 and PM-20 did not Modify mGluR-LTD

Pharmacokinetic studies have shown that LP-211 and BA-10 are metabolized by liver enzymes, respectively giving rise to the compouds RA-7 (Leopoldo et al., 2008) and PM-20 (Prof. Leopoldo, unpublished results). A previous study showed that RA-7 and PM-20 bind 5-HT$_7$ receptors with higher affinity than LP-211 (Lacivita et al., 2012). Similar to LP-211, RA-7 is brain penetrant and induced hypothermia in wild-type but not in 5-HT$_7$ KO mice following *in vivo* administration (Hedlund et al., 2010). These results suggest that RA-7 might be able to activate 5-HT$_7$ receptors. Therefore, we tested the effects of RA-7 on mGluR-LTD. In our experimental protocol, application of RA-7 (10 nM, 5 min) after DHPG application did not reverse mGluR-LTD (**Figure 4A**): EPSC amplitude measured 45 min after DHPG application was significantly reduced with respect to control (64 ± 7% of control EPSC; mean ± SEM, $n = 5$; $P<0.01$). The amount of mGluR-LTD following RA-7 application was not significantly different from mGluR-LTD in control conditions ($P = 0.5$, **Figure 4C**).

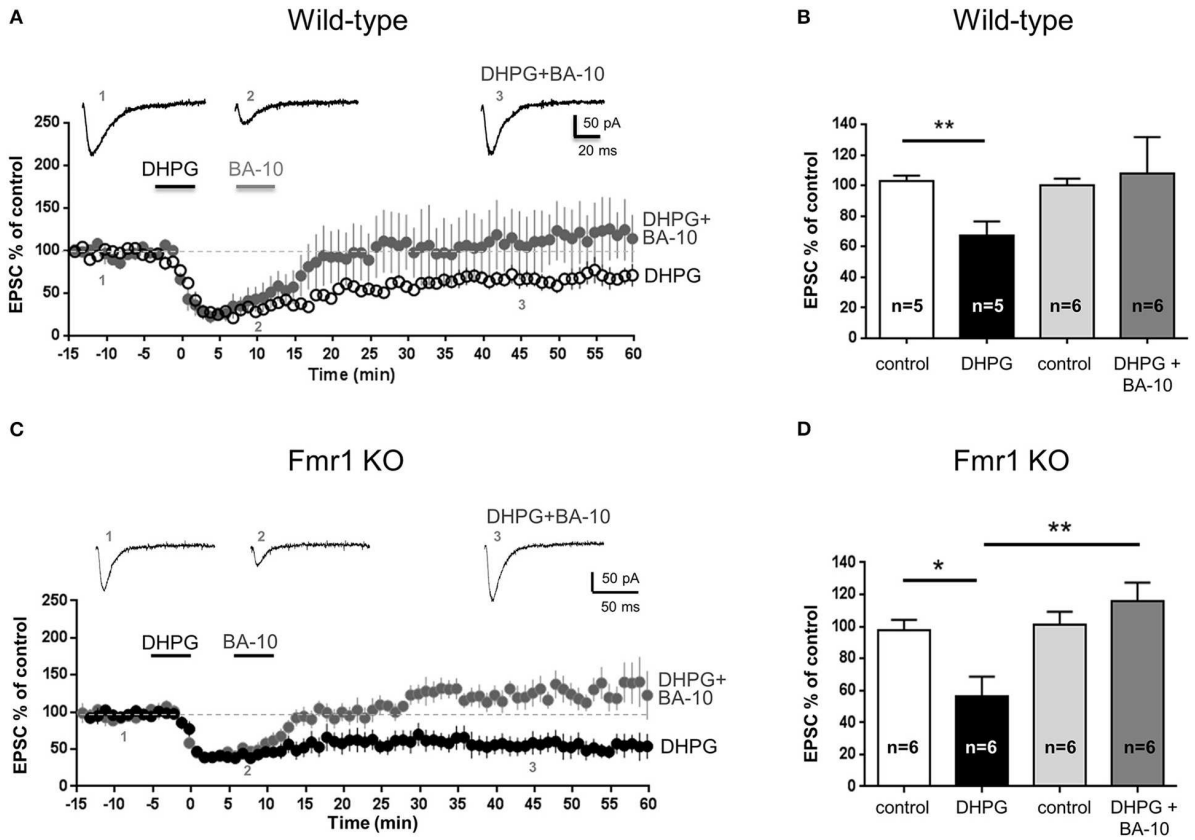


FIGURE 3 | The novel 5-HT$_7$ receptor ligand BA-10 reversed mGluR-LTD in the hippocampus of wild-type and Fmr1 KO mice Pooled data of % EPSC values (mean ± SEM) were plotted vs. time; insets show individual EPSC traces from representative experiments. mGluR-LTD was chemically induced by bath application of DHPG (100 μM, 5 min). **(A,B)** In hippocampal slices (300 μm) from wild-type mice (FVB; PN 14-21), application of DHPG induced a significant mGluR-LTD (**$P = 0.007$, $n = 5$). When LTD induction was followed by application of BA-10 (10 nM, 5 min), EPSC amplitude was not significantly different from control ($P = 0.75$, $n = 6$), indicating that mGluR-LTD was completely reversed. This result shows that BA-10 behaved as an agonist of 5-HT$_7$ receptors. **(C,D)** In slices from Fmr1 KO mice (C57BL6J, PN 14-20), application of DHPG (100 μM, 5 min) induced a significant mGluR-LTD (*$P = 0.010$, $n = 6$) that was completely reversed when BA-10 (10 nM, 5 min) was applied after DHPG ($P = 0.29$, $n = 6$). The amount of LTD induced by DHPG in the absence and in the presence of BA-10 was significantly different (**$P = 0.0054$). This result shows that BA-10 was able to reverse mGluR-LTD in a mouse model of Fragile X Syndrome.

In a similar way, application of PM-20 did not reverse DHPG-induced mGluR-LTD (**Figure 4B**): EPSC amplitude measured 45 min after DHPG application was significantly lower than control (**Figure 4C**, EPSC amplitude 60 ± 7% of control, mean ± SEM, $n = 5$; $P = 0.001$). The amount of mGluR-LTD induced by DHPG followed by PM-20 was not significantly different from mGluR-LTD induced by application of DHPG alone ($P = 0.9$, **Figure 4C**)

These results indicate that RA-7 and PM-20 were unable to induce the same effect of their parent compounds LP-211 and BA-10, thus did not behave as agonists of 5-HT$_7$ receptors in our experimental conditions.

Discussion

In a previous work, we have shown that 5-HT, through activation of 5-HT$_7$ receptors, is able to reverse exaggerated mGluR-LTD in the Fmr1 KO mouse model of Fragile X Syndrome (FXS) (Costa et al., 2012a). Exaggerated mGluR-LTD in Fmr1 KO mice is considered an electrophysiological readout of abnormal signaling through mGlu receptors (Bhakar et al., 2012). Our current hypothesis is that activation of 5-HT$_7$ receptors, by correcting mGluR-mediated mechanisms in Fmr1 KO mice, besides reversing abnormal mGluR-LTD can also rescue other typical phenotypes of FXS, particularly abnormal dendrite morphology, cognitive impairment and autistic behavior (Osterweil et al., 2012; Ciranna and Catania, 2014). Interestingly, 5-HT$_7$ receptor activation was shown to stimulate neurite and dendrite outgrowth in cultured neurons and is believed to play a crucial role in brain wiring during development (Volpicelli et al., 2014). These data suggest that activation of 5-HT$_7$ receptors might also correct abnormal dendrite morphology in Fmr1 KO mice.

To test this hypothesis, selective and high-affinity 5-HT$_7$ receptor agonists are needed: most importantly, the new agonists must also display drug-like properties and reach the brain following *in vivo* systemic administration. On this purpose,

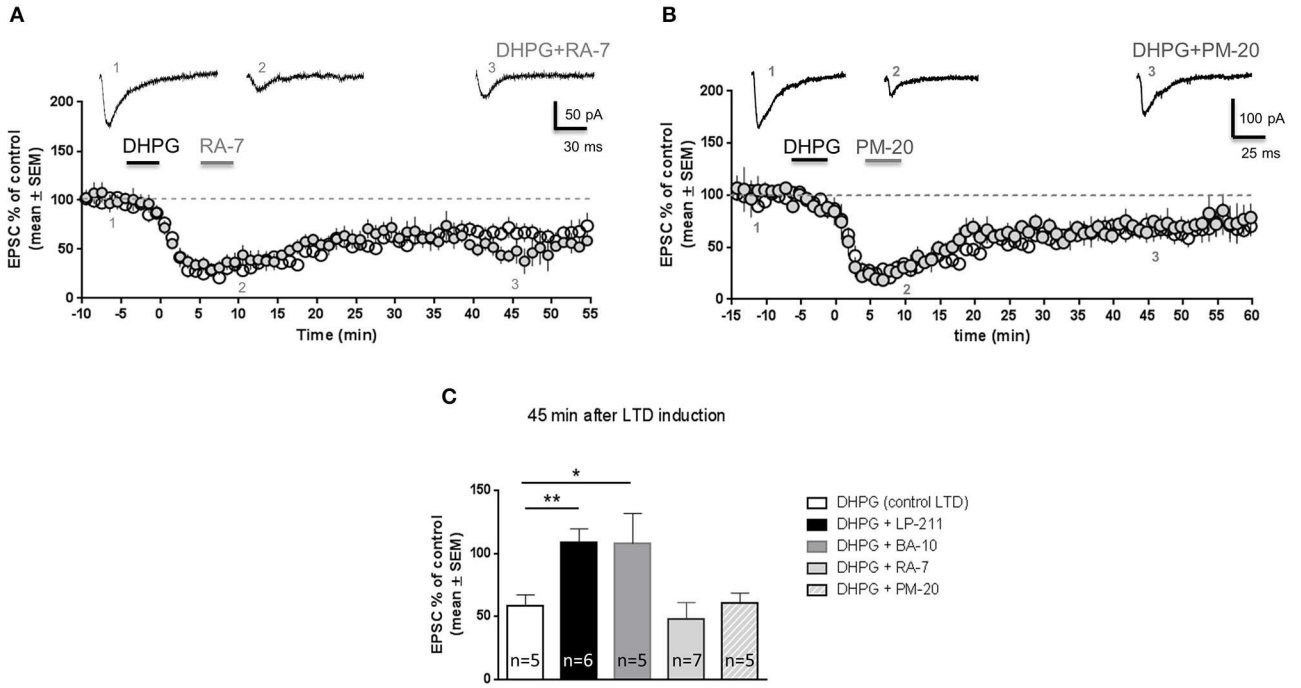


FIGURE 4 | RA-7 and PM-20 did not reverse mGluR-LTD in the hippocampus of wild-type mice. Hippocampal slices (300 μm) were prepared from wild-type mice (FVB; PN 14-21). Pooled data of % EPSC values (mean ± SEM) were plotted vs time; insets show individual EPSC traces from representative experiments. When DHPG application was followed by application of RA-7 (**A;** 10 nM, 5 min) or PM-20 (**B;** 10 nM, 5 min), mGluR-LTD was not reversed. **(C)** Histograms show mean EPSC amplitude (% of control) 45 min after DHPG application (empty column, $n = 5$); when DHPG application was followed by application of either LP-211 (black column, $n = 6$) or BA-10 (dark gray column, $n = 5$), EPSC amplitude was significantly higher than following application of DHPG alone ($^{**}P < 0.001$; $^{*}P < 0.01$ vs. DHPG alone, Student's *t*-test), indicating that mGluR-LTD was reversed. Vice-versa, RA-7 and PM-20 were unable to reverse mGluR-LTD (no significant difference vs. DHPG alone), thus did not behave as agonists of 5-HT_7 receptors.

in the present work we have tested the effects of a brain-permeant 5-HT_7 receptor agonist (LP-211) and characterized the pharmacokinetic properties and biological effects of a novel putative 5-HT_7 receptor agonist (BA-10). We show that both substances are able to correct a synaptic malfunction in Fmr1 KO mice, thus can be used for *in vivo* administration to Fmr1 KO mice in preclinical behavioral studies.

LP-211 was designed and synthesized by the research group of Prof. Leopoldo (compound 25 in (Leopoldo et al., 2008) and characterized as a high-affinity, selective and brain-permeant 5-HT_7 receptor agonist (Hedlund et al., 2010). *In vivo* administration of LP-211 was shown to exert pro-cognitive effects in rats submitted to an autoshaping learning task (Meneses et al., 2015).

Our previous results (Costa et al., 2012a) demonstrated that LP-211 is able to reverse mGluR-LTD in wild-type mice. Here we show that LP-211 reverses mGluR-LTD also in Fmr1KO mice, in which mGluR-LTD is abnormally enhanced, suggesting a potential therapeutic use of LP-211 in Fragile X Syndrome.

In slices from Fmr1KO mice, reversal of mGluR-LTD by LP-211 showed a slower time-course with respect to wt. In addition we observed that, unlike in wt, in Fmr1 KO mice LP-211 did not fully reverse mGluR-LTD but rather restored LTD level comparable to wt condition. This result is in agreement with our previous observation that in Fmr1 KO mice 8-OH-DPAT partially reduced the amount of mGluR-LTD and did not completely abolish it (Costa et al., 2012a). This might have important functional consequences, since long-term synaptic plasticity plays a fundamental role in shaping the structure and function of brain circuits. A large body of evidence demonstrates that LTD is crucially involved in learning and memory and pharmacological or genetic manipulations disrupting LTD also impair learning (Collingridge et al., 2010). Consistently, abnormal mGluR-LTD has been observed in animal models of several cognitive diseases including Fragile X Syndrome (D'Antoni et al., 2014). Interestingly, LTD plays a crucial role in novelty detection and in the extinction of previously acquired memories and is believed to underlie behavioral flexibility (Collingridge et al., 2010). A reduced behavioral flexibility, i.e., a reduced ability to face a new environmental context, is a typical feature of autism spectrum disorders. Fragile X Syndrome is a leading genetic cause of autism (Hagerman et al., 2005) and alterations in behavioral flexibility have been described in FXS patients (Hooper et al., 2008) as well as in Fmr1KO mice (Bernardet and Crusio, 2006; Casten et al., 2011; Krueger et al., 2011). We suggest that selective activation of 5-HT_7 receptors, by restoring mGluR-mediated synaptic plasticity to normal levels, might also rescue cognitive functions and behavioral flexibility in the mouse model of Fragile X Syndrome (Ciranna and Catania, 2014). We will test this hypothesis in

the near future by behavioral studies on wt and Fmr1 KO mice following *in vivo* administration of a 5-HT_7 receptor agonist.

As already pointed out, LP-211 is suitable as a centrally-active substance following systemic administration, being able to pass the blood brain barrier (Hedlund et al., 2010), but showed a relatively short *in vivo* half-life (65 min) when administered to mice by intraperitoneal injection (Leopoldo et al., 2008). In the attempt to identify a 5-HT_7 receptor agonist with improved pharmacokinetic properties, we have designed and characterized a series of new molecules structurally related to LP-211.

Among the novel compounds examined in the present work, BA-10 displayed the most suitable pharmacokinetic properties for systemic administration. Vice-versa, the compounds MM-1 and MM-2 displayed lower *in vitro* metabolic stability compared to LP-211 and were not further considered for our study. As for compound PF-62, *in vitro* pharmacokinetic properties did not show any improvement with respect to LP-211. On the other side, BA-10 displayed higher selectivity on 5-HT_7 receptors and improved metabolic stability with respect to LP-211. We show that BA-10 significantly reversed mGluR-LTD (an effect mediated by 5-HT through activation of 5-HT_7 receptors, as we have previously characterized) in the hippocampus of wild-type and Fmr1 KO mice. These results demonstrate that BA-10 behaves as an agonist of 5-HT_7 receptors and is able to correct exaggerated mGluR-LTD in the mouse model of Fragile X Syndrome. In slices from Fmr1 KO mice, reversal of mGluR-LTD by BA-10 at a 10 nM dose was more significant than the effect induced by LP-211 at the same concentration (compare **Figures 2C,D** and **Figures 3C,D**). The higher effectiveness of BA-10 with respect to LP-211 is in agreement with a 10 fold higher affinity of BA-10 vs. LP-211 for 5-HT_7 receptors, as we measured by radioligand binding assays (see **Table 1**), and indicates that BA-10 can be used at very low (sub-nanomolar) doses to activate 5-HT_7 receptors.

We observed that BA-10 showed a weak *in vitro* affinity for interaction with P-glycoprotein, suggesting that BA-10 might persist in CNS without being extruded by the blood brain barrier when administered systemically. Consistently, *in vivo* results recently published (Hansen et al., 2014) show that BA-10 is not significantly transported by P-glycoprotein and reaches high brain concentrations following intravenous injection. Therefore, the pharmacokinetic properties of BA-10 are promising and deserve to be further investigated.

RA-7 and PM-20, metabolites of LP-211 and BA-10 respectively, were unable to reverse mGluR-LTD, thus did not behave as agonists of 5-HT_7 receptors in our experimental protocol. This indicates that, following *in vivo* administration of LP-211 and BA-10, their metabolites RA-7 and PM-20 do not exert a cooperative action with parent compounds, as was hypothesized for RA-7 (Hedlund et al., 2010). The lack of agonist effect of RA-7 and PM-20 also suggests that, following *in vivo* administration, the effects of LP-211 and BA-10 are likely to be reduced with time as their concentration is decreased by hepatic metabolism. Nevertheless, several results indicate that LP-211 exerts long-term effects on the brain. The first pharmacokinetic study on LP-211 (Leopoldo et al., 2008) shows that the brain concentration of LP-211 in mice remained high during at least 2 h after intraperitoneal injection. The authors suggested that LP-211 is likely to be accumulated in the brain, due to its high lipophilic properties. Besides, it should be noticed that 2 h after injection, LP-211 brain concentration declined below detectable levels, but might still be high enough to activate 5-HT_7 receptors, especially considering that LP-211 binds 5-HT_7 receptors with very high affinity, with reported Ki values between 0.58 nM (Leopoldo et al., 2008) and 15 nM (Hedlund et al., 2010). In line with this, we observed a reversal of mGluR-LTD by a low nanomolar dose of LP-211 (10 nM). Consistently, LP-211 has been administered *in vivo* in several studies and was shown to exert long-term effects on functions regulated by the central nervous system such as regulation of body temperature, sleep and circadian rhythms (Hedlund et al., 2010; Adriani et al., 2012; Monti et al., 2014). These results suggest that central effects of LP-211 can be long-lasting in spite of a relatively short half-life.

In conclusion, we show that the 5-HT_7 receptor agonists LP-211 and BA-10 correct a synaptic malfunction in Fmr1KO mice, thus might become new pharmacological tools for the therapy of Fragile X Syndrome. We also show that the novel compound BA-10 is a highly effective 5-HT_7 receptor agonist with improved selectivity and *in vitro* pharmacokinetic properties with respect to LP-211, thus should also be considered for *in vivo* studies.

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Long-lasting beneficial effects of central serotonin receptor 7 stimulation in female mice modeling Rett syndrome

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Rett syndrome (RTT) is a rare neurodevelopmental disorder, characterized by severe behavioral and physiological symptoms. Mutations in the methyl CpG binding protein 2 gene (*MECP2*) cause more than 95% of classic cases, and currently there is no cure for this devastating disorder. Recently we have demonstrated that specific behavioral and brain molecular alterations can be rescued in MeCP2-308 male mice, a RTT mouse model, by pharmacological stimulation of the brain serotonin receptor 7 (5-HT7R). This member of the serotonin receptor family—crucially involved in the regulation of brain structural plasticity and cognitive processes—can be stimulated by systemic repeated treatment with LP-211, a brain-penetrant selective 5-HT7R agonist. The present study extends previous findings by demonstrating that the LP-211 treatment (0.25 mg/kg, once per day for 7 days) rescues RTT-related phenotypic alterations, motor coordination (*Dowel test)*, spatial reference memory (*Barnes maze test*) and synaptic plasticity (*hippocampal long-term-potentiation*) in MeCP2-308 heterozygous female mice, the genetic and hormonal milieu that resembles that of RTT patients. LP-211 also restores the activation of the ribosomal protein (rp) S6, the downstream target of mTOR and S6 kinase, in the hippocampus of RTT female mice. Notably, the beneficial effects on neurobehavioral and molecular parameters of a seven-day long treatment with LP-211 were evident up to 2 months after the last injection, thus suggesting long-lasting effects on RTT-related impairments. Taken together with our previous study, these results provide compelling preclinical evidence of the potential therapeutic value for RTT of a pharmacological approach targeting the brain 5-HT7R.

Keywords: serotonin, neurodevelopmental disorders, intellectual disability, transgenic mice, synaptic plasticity, Rho GTPases, cognition

Introduction

The serotonin receptor 7 (5-HT7R), coded by Htr7 gene, is among the most recently discovered serotonin receptors (Barnes and Sharp, 1999). This seven-transmembrane and G protein-coupled

receptor is characterized by a widespread expression in the central nervous system and in the periphery (Romano et al., 2014). The highest 5-HT7 receptor density in the brain is found in the thalamus and hypothalamus, as well as in the hippocampus (Guseva et al., 2014). Thanks to the availability of pharmacological and genetic tools targeting the 5-HT7R in preclinical models (Leopoldo et al., 2011; Matthys et al., 2011), a link with neuro-physiological phenomena like regulation of the circadian rhythm, sleep, mood and thermoregulation has been clearly established (Matthys et al., 2011; Adriani et al., 2012; Monti and Jantos, 2014). The relevance of the 5-HT7R in various psychiatric and neurological disorders, such as anxiety, schizophrenia, pain and epilepsy has been also addressed (Hedlund, 2009; Di Pilato et al., 2014). An increasing number of studies demonstrates a role for the 5-HT7R on cognitive processes (particularly on hippocampal-dependent learning and memory) and in the regulation of structural plasticity in adolescent and mature brain circuits (Gasbarri and Pompili, 2014; Meneses, 2014; Volpicelli et al., 2014; Canese et al., 2015). Consistent with these observations, the 5-HT7R activation stimulates signaling cascades known to play a prominent role in synaptic plasticity and cognition, with the more prominent downstream effectors being represented by the extracellular-signal regulated kinases (ERKs), the cyclic AMP protein kinase (PKA) and the Cyclin-dependent kinase 5 (Cdk5; Guseva et al., 2014; Volpicelli et al., 2014).

In cultured hippocampal neurons, receptor-mediated activation of the Gα12 signaling pathway also results in the selective activation of small Rho GTPases (Kvachnina et al., 2005; Kobe et al., 2012), a family of proteins crucially involved in neuronal plasticity and cognition and key regulators of actin cytoskeleton dynamics (De Filippis et al., 2014b). The 5-HT7R-mediated stimulation of this signaling pathway leads to pronounced changes in neuronal morphology and plasticity (Kobe et al., 2012), thus providing further support to a crucial involvement of 5-HT7R in the regulation of neurobiological mechanisms underlying cognitive functions (Volpicelli et al., 2014). Recently, we have extended these findings by providing the first *in vivo* evidence that a pharmacological stimulation of the 5-HT7R (i.e., by systemic administration of LP-211, a novel selective and brain penetrant 5-HT7R agonist: see (Hedlund et al., 2010; Romano et al., 2014), activates Rho GTPases in mouse brain (De Filippis et al., 2014a). Given the involvement of brain Rho GTPases in a number of neurological disorders and the paucity of drugs targeting this family of proteins *in vivo* (De Filippis et al., 2014b), these data pointed to LP-211 as an innovative pharmacological tool to be exploited in preclinical research. In this line, activation of 5-HT7Rs has been reported to reverse electrophysiological abnormalities in hippocampal slices collected from a mouse model of Fragile X syndrome (Costa et al., 2012).

Based on these findings, we have recently investigated the potential therapeutic value of a pharmacological stimulation of the central 5-HT7R for Rett syndrome (RTT), a rare and severe neurodevelopmental disorder (Percy and Lane, 2005) and one of the leading causes of mental disability in girls (Rett, 1966; Hagberg, 2002). RTT patients present stereotypical hand movements, limited language, severe autistic−like features and intellectual disability, as well as potentially life−threatening seizures and respiratory dysfunction. Mutations in the methyl-CpG-binding protein 2 (MeCP2) gene, located on Xq28, have been identified as the main genetic cause of RTT (Amir et al., 1999) and account for more than 95% of classic RTT (Chahrour and Zoghbi, 2007). At present there is no cure for RTT and available treatments are symptomatic.

Consistent with previous evidence pointing to Rho GTPases as therapeutic targets for RTT (De Filippis et al., 2012) and with previous studies suggesting that serotonergic neurotransmission is deeply affected both in RTT patients and animal models (Isoda et al., 2010; Santos et al., 2010; Moroto et al., 2013), we demonstrated that a seven-day-long treatment with LP-211 reverses anxiety-related profiles in a Light/Dark test, motor abilities in a Dowel test, the exploratory behavior in the Marble Burying test, as well as short-term working memory in the Y maze task in MeCP2-308 hemizygous (hz) male mice (Ricceri et al., 2013). Moreover, this treatment was found to restore the activation of Rho GTPases effector molecules, PAK, cofilin and the ribosomal protein (rp) S6, a downstream target of mTOR and S6 kinase (Ricciardi et al., 2011), in RTT mouse hippocampus (De Filippis et al., 2014a).

In the present study, we aimed at assessing whether the beneficial effects of the LP-211 treatment in RTT mice extend beyond the behavioral domains we had previously investigated (De Filippis et al., 2014a). To increase the translational value of the study, we focused on MeCP2-308 heterozygous (Het) female mice, given that their genetic and hormonal milieu more closely resembles that of RTT patients (Katz et al., 2012). Increasing studies in fact demonstrate that heterozygosity does not preclude the use of female mice in RTT preclinical studies (Woods et al., 2012; Garg et al., 2013). Therefore, in symptomatic MeCP2-308 Het female mice, we have evaluated whether treatment with LP-211 affects spatial reference memory deficits in the Barnes Maze test and impairments of hippocampal long-term potentiation (LTP), a form of synaptic plasticity thought to underline long-term memory formation. Given the demonstrated role played by 5-HT7R in the regulation of cognitive performance and synaptic plasticity (Gasbarri and Pompili, 2014; Meneses, 2014; Volpicelli et al., 2014), we argued that 5-HT7R stimulation by LP-211 in RTT mouse brain might restore these additional RTT-related deficits. Moreover, in order to confirm data obtained in LP-211-treated MeCP2-308 male mice (De Filippis et al., 2014a) and assess whether this treatment is equally effective under conditions of heterozygosis, the effects of the treatment on motor coordination deficits in RTT female mice were also addressed with the Dowel test.

Another major aim of the present study was to discover whether a seven-day-long treatment with LP-211 may produce long-lasting changes in the neurobehavioral phenotype or at the brain molecular level in RTT mice. This hypothesis stems from previous studies demonstrating that transient modulation of Rho GTPases in RTT mouse brain produces long-lasting

beneficial effects (De Filippis et al., 2012) and that 5-HT7 stimulation during periods of increased plasticity, such as adolescent age, may result in neuro-plastic changes leading to a persistent modification on forebrain circuits (Adriani et al., 2006; Altabella et al., 2014; Canese et al., 2015). To evaluate this hypothesis, we applied the seven-day-long treatment schedule known to induce Rho GTPases activation in mouse brain (De Filippis et al., 2014a) and tested the effects on general health parameters as well as behavioral, molecular and synaptic plasticity endpoints up to 2 months after the last injection).

Materials and Methods

Subjects

The experimental subjects were 1-year old MeCP2-308 heterozygous female mice [B6.129S-MeCP2tm1Heto/J, stock number: 005439; backcrossed to C57BL/6J mice for at least 12 generations from the Jackson Laboratories (USA)] and wild-type (wt) littermates. The MeCP2-308 model bears a truncating mutation, leading to the expression of a protein truncated at amino acid 308 (Shahbazian et al., 2002). In agreement with clinical data from RTT patients carrying C-terminal deletions of the MeCP2 gene (Díaz de León-Guerrero et al., 2011), this model presents a delayed onset of symptoms and a prolonged life-span in comparison with knockout mice (Ricceri et al., 2008).

Mice were housed in groups of 2–3 in polycarbonate transparent cages (33 × 13 × 14 cm) with sawdust bedding and kept on a 12-h light-dark schedule (lights off at 8:00). Temperature was maintained at 21 ± 1°C and relative humidity at 60 ± 10%. Animals were provided *ad libitum* with tap water and a complete pellet diet (Altromin, Germany). All procedures were carried out in accordance with the European Communities Council Directive (86/609/EEC) as well as Italian law, and formally approved by Italian Ministry of Health.

Drug and Treatment

LP-211 was prepared following the same synthetic procedure described in Leopoldo et al. (2008). The compound was dissolved in a vehicle solution of 1% dimethyl sulfoxide (DMSO) in saline (0.9% NaCl). MeCP2-mutated mice and wt littermate controls were randomly assigned to be daily intra-peritoneally (ip) injected (between 9.00 and 11.00 am) for 7 consecutive days with either LP-211 (0.25 mg/kg) or vehicle (1% of DMSO in saline). The number of mice for each condition was as follows: wt, Veh = 9; wt, LP-211 = 11; Het, Veh = 12; Het, LP-211 = 10.

To test whether LP-211 can counteract RTT related abnormalities when they are fully manifested, MeCP2-308 heterozygous female mice were treated at about 1 year of age, when abnormalities have been reported to start appearing (Shahbazian et al., 2002).

Behavioral Testing

Mice were experimentally naïve at the start of the test battery. All behavioral testing took place during the dark phase of the L/D cycle, between 9.00 am and 6.00 pm, and was carried out by experimenters blind to mouse genotypes and treatments. The estrous cycle was not controlled in this study (Prendergast et al., 2014). A minimum of 24 h was left between each test, as follows: the *Dowel test* was performed 2 h after the 7th ip injection; the *open field test* was carried out 24 h after the last ip injection (on the 8th day of the schedule); the *general health scoring* was carried out after the *open field test* on the 8th day of the schedule and 23 days after the last ip injection (on the 30th day of the schedule); the training on the *Barnes Maze test* started on the 14th day of the schedule and the probe tests were conducted on the 21st and 28th days of the schedule (see **Figure 1** for experimental design and treatment schedule).

General Health Scoring

The general health of the experimental mice was qualitatively evaluated by a trained observer as previously described (Guy et al., 2007; De Filippis et al., 2014a), with little modification. Briefly, mice received a score (ranging from 0: normal appearance, to 2: highly compromised) for each of the following symptoms: gait, mobility, breathing, kyphosis, fur, hindlimb clasping, tremors, general condition. The individual scores for

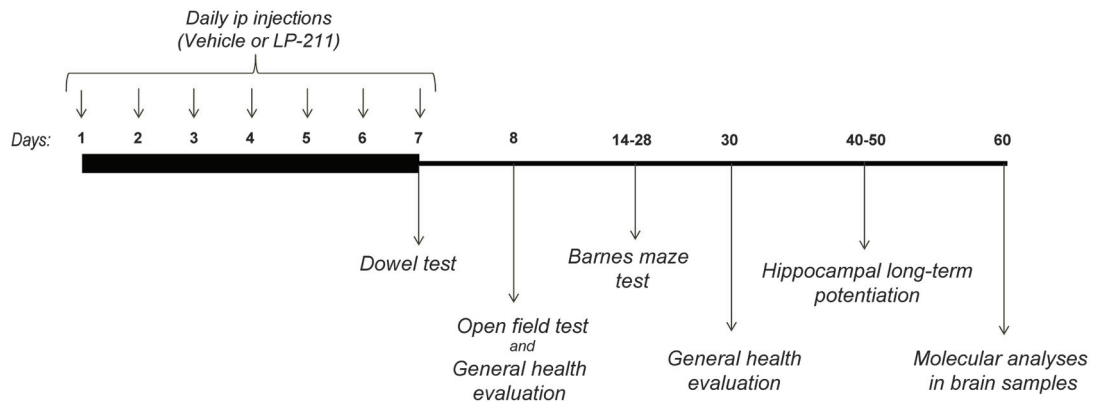

FIGURE 1 | Experimental Design.

each of these categories were subsequently averaged to obtain a semi-quantitative measure of individual symptom status, called throughout the text "*the general health score*".

Body weight and body (rectal) temperature were recorded after each general health scoring.

Dowel Test

To evaluate the effects of the LP-211 treatment on motor coordination capacities, the *Dowel test* was performed as previously described (De Filippis et al., 2014a). The hardwood round dowel used was 9.0 mm in diameter and 35 cm long. The dowel was mounted horizontally 50 cm above a 5 cm depth bedding of sawdust. At the beginning of the each testing session, each mouse was placed in the middle of the dowel so that the length of its body was parallel to it. Latency to fall from the dowel into a cage of bedding was recorded (30-s criterion). Each mouse repeated the test three times, with an intertrial interval of at least 15 min. If mice were able to walk across the dowel and off of the dowel, they received the maximum score of 30 s.

Open Field Test

The *open field* apparatus was a gray plastic box (40 × 40 cm) surrounded by high walls (35 cm). Each mouse was individually placed in the *open field* and allowed to freely explore the environment for a 60 min session (De Filippis et al., 2013). The floor of the apparatus was cleaned with 20% ethanol after each animal was tested and the test was carried out under dim lights. Three intervals of 5 min (0–5; 30–35; 55–60) were subsequently scored by a trained observer blind to the genotype and treatment of mice, using a computer and a specific software (THE OBSERVER v2.0 for DOS, Noldus Information Technology, Wageningen, Netherlands). The floor of the apparatus was subdivided into 16 sections (10 × 10 cm) by lines placed on the video screen at the time of videotape analysis. The frequency and durations of the following items were scored: *Crossing* (number of line crossings with both forepaws), *Rearing* (body in vertical position), *Wall rearing* (body in vertical position with forepaws placed on the walls of the cage), *Grooming* (mouth or paws on body) and *Inactivity* (complete absence of movements including small movements of head, ears or vibrissae).

Barnes Maze Test

To assess whether the treatment with LP-211 has beneficial effects on spatial reference memory deficits in RTT mice, the *Barnes Maze test* was carried out as previously described (Barnes, 1979).

In this test, mice were trained to locate a black rectangular escape box (7 × 37 × 9 cm) hidden underneath one of 12 holes (4 cm in diameter) evenly spaced around the perimeter of an elevated (36 cm above the floor) gray platform (95 cm in diameter), illuminated by overhead fluorescent white room lighting (85 lux). The hole above the escape box was designated as the target, analogous to the hidden platform in the Morris water maze task. The location of the target was consistent for a given mouse but randomized across mice. To prevent orientation to the target before a trial began, mice were initially placed in the center of the platform under a black cylinder (12 cm in diameter). The cylinder was removed after 10 s and the trial begun. The maze was cleaned with a 20% ethanol solution between each trial.

Behavioral testing consisted of an adaptation period, an acquisition phase and 2 probe trials. During the adaptation phase (14th day of the schedule), mice were allowed to explore for two consecutive daily trials the platform. During each trial, if 1 min had elapsed without the mice entering the target hole, they were placed to the side of the target hole and gently helped to enter the escape box. Once inside the escape box, the mouse was left there for 2 min. No parameters were recorded during this phase.

During the acquisition phase (from the 15th to the 20th day of the schedule), mice were given two trials per day during which latency to enter the target hole and total path length were recorded. When the trial ended (i.e., when the mouse entered the escape box or after 3 min had elapsed), the mouse was left inside the escape box for 1 min. An intertrial interval of at least 10 min was used. During the acquisition phase, latency, errors and path length to enter the target hole were measured. For statistical analyses parameters were averaged in blocks of trials per day (mean ± S.E.M).

The first probe trial was conducted 24 h after the last training trial to assess short-term reference memory retention (21th day of the schedule). To assess long-term retention a second probe trial was applied 7 days after the first probe (28th day of the schedule). No training trials were conducted between the two probe tests. During the probe tests, the target hole was closed to confirm that mice used only extra-maze cues to reach the escape box. A 90-s long session was used, during which primary latency (to first nose poke in the virtual target hole) and primary errors (i.e., nose pokes before arriving to the target) were measured.

Neurobiological Analyses

Starting on the 40th day of the schedule, mice underwent electrophysiology experiments (N = 4 mice per experimental group). The brains of the remaining subjects were dissected and rapidly frozen for biochemical analyses at sacrifice, 2 months after the last ip injection.

Hippocampal Slice Electrophysiology

To evaluate the induction of LTP in wt and MeCP2-308 female mice, hippocampal slices were prepared as previously described (Domenici et al., 2006). Field excitatory post-synaptic potentials (fEPSPs) were recorded in stratum radiatum of CA1 area after stimulation of the Schaffer collaterals. Traces were acquired, amplified and analyzed with DAM-80 AC differential amplifier (WPI Instruments) and with the WinLTP software (Anderson and Collingridge, 2007). Stimuli (100 μs duration) were set to an intensity that evokes a fEPSP with a slope of 60% of the maximum fEPSP slope and delivered every 20 s (three consecutive responses were averaged). LTP was induced by a theta-burst stimulation (TBS) consisting in 2 trains of 5 sets of bursts (four stimuli, 100 Hz) with an interburst interval of 200 ms and a 20 s interval between each train. Synaptic transmission was recorded for 60 min and 10 min of stable baseline recordings preceded LTP induction. Changes in fEPSP slope were expressed as percentage changes with respect to the average slope of the fEPSP measured during the 10 min that preceded the TBS.

Western Blot Analyses

Proteins were analyzed by western blotting as previously described (De Filippis et al., 2014a). Briefly, hippocampal tissues were isolated and homogenized in lysis buffer immediately after sacrifice, proteins were separated by SDS-PAGE and blotted to nitrocellulose membrane. The following primary antibodies were used: rabbit polyconal anti-rpS6 (1:1000, cod. 2217, Cell Signaling), rabbit polyclonal anti-phospho-rpS6 (1:200, cod. 2211 (Ser 235/236) and cod. 2215 (Ser 240/244), Cell Signaling), mouse monoclonal anti β-actin (1:1000, cod. sc-81178, Santa Cruz). Optical densities (O.D.) of the protein signals from at least three different experiments were calculated for each sample and normalized with the corresponding β-actin signal; the O.D. ratios were then compared and expressed as the average fold increase, with 1 (wt control) as baseline.

Statistical Analysis

Data were analyzed with either parametric or non-parametric analysis of variance, depending on distribution of the response variable considered. The Shapiro-Wilk test was applied to verify the normality of data distribution. ANOVA models included genotype (wt vs. Het) and treatment (vehicle vs. LP-211) as between-subject factors, and repeated measurements as within-subject factor. *Post hoc* comparisons were run using Tukey's test, which can be performed also in the absence of significant ANOVA results (Wilcox, 1987). The Levene test was applied to confirm that variance did not differ between groups. To unravel the presence of outliers, the Grubbs' test was applied.

Results

General Health Scoring

Kruskall Wallis analyses confirmed that significant differences among the experimental groups are present on both time points under investigation (first time interval: $H = 8.89$; $p = 0.031$; second time interval: $H = 9.31$; $p = 0.025$; **Figure 2**). *Post hoc* comparisons, carried out with the Mann Whitney U test, revealed that—at 24 h after the last ip injection—slight but reliable phenotypic alterations can be detected in MeCP2-308 female mice compared to wt controls (wt, Veh vs. Het, Veh: $U = 23.50$; $p = 0.030$; **Figure 2**). In particular, the phenotypic parameters which appeared most compromised in MeCP2-308 female mice were the gait, the kyphosis and breathing. Contrary to MeCP2-null mice (Guy et al., 2007), no hindlimb clasping or abnormal mobility were observed in RTT mice vs. wt controls. At this point of the treatment schedule (**Figure 1**), no recovery in the general health status was observed for RTT mice treated with LP-211 compared to vehicle, with both being significantly worse than wt controls (**Figure 2**). Notably, however, recovery was observed 3 weeks later: MeCP2-308 female mice that had received the vehicle treatment were slightly worsened (wt, Veh vs. Het, Veh: $U = 18.00$; $p = 0.011$), whereas RTT mice that were treated with LP-211 exhibited an improved general health status (Het, Veh vs. Het, LP-211: $U = 29.00$; $p = 0.041$; **Figure 2**). A delayed effect of the 7-day long LP-211 treatment on general health status of RTT mice was thus demonstrated.

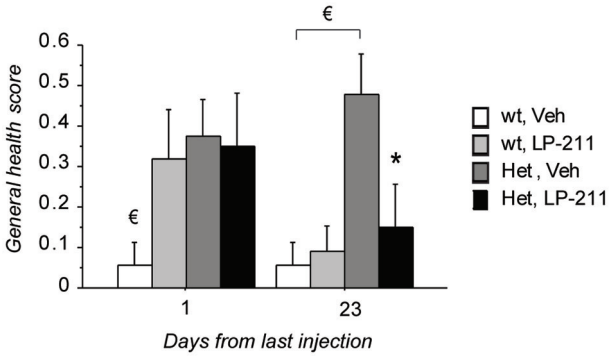

FIGURE 2 | Treatment with LP-211 exerts delayed beneficial effects on general health parameters in MeCP2-308 female mice. MeCP2-308 female mice (Het) showed slight but reliable phenotypic alterations compared to wt mice in control conditions (Veh). The seven-day long LP-211 treatment improved the general health status selectively in Het mice (Het, LP-211) after 3 weeks from the last injection. Mice for each condition were as follows: wt, Veh = 9; wt, LP-211 = 11; Het, Veh = 12; Het, LP-211 = 10. Data are mean ± SEM. € : wt, Veh vs. Het, Veh, $p < 0.05$ and * : Het, Veh vs. Het, LP-211, $p < 0.05$.

No significant genotype nor treatment effects were found as for body weight and body temperature (data not shown).

Dowel Test

Performance in the Dowel test on the 7th day of treatment was investigated to confirm data obtained in LP-211-treated MeCP2-308 hz male mice (De Filippis et al., 2014a). The repeated measures (RM)- ANOVA yielded a significant main effect of genotype ($F_{(1,38)} = 4.12$; $p = 0.049$), with RTT mice falling from the dowel significantly earlier than wt controls (**Figure 3**), and a genotype by treatment interaction just missing significance ($F_{(1,38)} = 3.77$; $p = 0.060$). *Post hoc* comparisons by Tukey's test confirmed that the performance of MeCP2-308 heterozygous female mice was worse compared to that of wt controls ($p < 0.05$). A just missing effect of the LP-211 treatment on the motor coordination ability of RTT mice was also found, with the treatment improving their performance (**Figure 3**). The LP-211 treatment did not affect the performance of wt mice.

Open Field Test

In order to clarify LP-211 treatment effects on general locomotor activity, MeCP2-308 mice and wt littermates were tested in the *open field* test. The RM- ANOVA did not highlight any difference between groups as for the mean number of *crossings* (wt Veh: 74.78 ± 5.69; wt LP-211: 69.67 ± 4.80; Het Veh: 79.53 ± 5.04; Het LP-211: 75.47 ± 4.63) and *the time mice spent in the central area* of the arena (wt Veh: 29.26 ± 7.18s; wt LP-211: 26.75 ± 5.81; Het Veh: 27.06 ± 3.92; Het LP-211: 22.75 ± 4.20). Mice also spent a similar amount of time being *immobile* or performing self-directed *grooming* (data not shown). Mutant mice however performed a significantly lower number of *rearings* (wt $_{(veh + LP\text{-}211\ pooled)}$: 34.88 ± 2.08; Het $_{(veh + LP\text{-}211\ pooled)}$: 25.59 ± 1.36; main effect of genotype: $F_{(1,38)} = 7.63$; $p = 0.009$), thus confirming previous reports (De Filippis et al., 2012). As a whole,

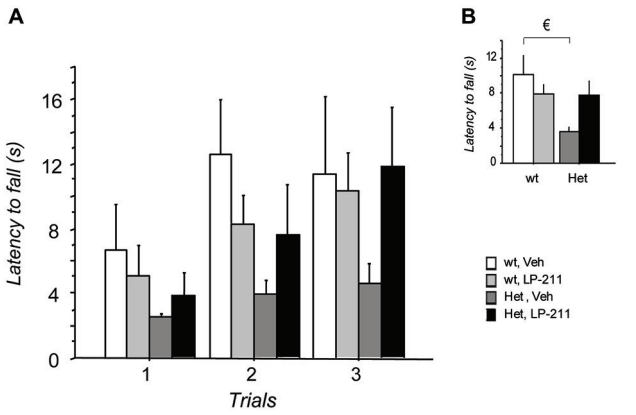

FIGURE 3 | Treatment with LP-211 improves motor coordination capacities of MeCP2-308 female mice in the Dowel test. (A) In the Dowel test, MeCP2-308 female mice (Het, Veh) displayed significantly shorter latencies to fall compared to wt controls (wt, Veh), confirming impaired motor coordination capacities. LP-211 improved the performance of Het mice in this test. Three trials per mouse were performed, with an intertrial interval of at least 15 min. **(B)** Data from the three trials were pooled to highlight significant differences between the experimental groups. Mice for each condition were as follows: wt, Veh = 9; wt, LP-211 = 11; Het, Veh = 12; Het, LP-211 = 10. Data are mean ± SEM. € : wt, Veh vs. Het, Veh, $p < 0.05$.

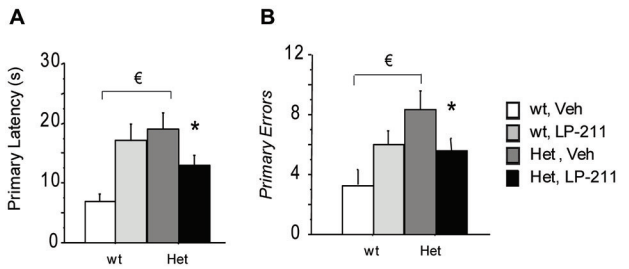

FIGURE 4 | Stimulation of the 5-HT7 receptor by LP-211 improves spatial memory of MeCP2-308 female mice in the Barnes Maze test. MeCP2-308 female mice (Het, Veh) displayed longer primary latencies **(A)** and a higher number of primary errors **(B)** to locate the virtual target hole (first nose poke in the virtual target hole) during the Probe tests. LP-211 treatment partially restored the levels of both parameters in Het mice **(A,B)**. Mice for each condition were as follows: wt, Veh = 9; wt, LP-211 = 11; Het, Veh = 12; Het, LP-211 = 10. Data are means ± SEMs. € : wt, Veh vs. Het Veh, $p < 0.05$ and * : Het, Veh vs. Het, LP-211, $p < 0.05$.

the LP-211 treatment did not affect the behavioral profile of either wt or mutant mice in this test.

Barnes Maze Test

Acquisition

As expected, latency to locate (first nose poke) and to enter the target hole decreased across trials during the acquisition phase (main effect of days: $F_{(4,148)} = 22.78$; $p > 0.001$; $F_{(4,148)} = 24.56$; $p > 0.001$, respectively), thus confirming that all the mice learned to locate the target hole across the training sessions.

As a whole, mutant mice did not differ from wt controls during the training trials (no genotype nor genotype by RM interactions were found for any of the analyzed parameters).

Probe Tests

On the 21st day (24 h after the last training trial) and the 28th day of the treatment schedule (7 days after the first probe test), the target hole was closed and the probe trials were conducted to assess spatial reference memory retention. Data were analyzed with the RM-ANOVA, with probe tests as within-subject factor. During both probe tests, MeCP2-308 heterozygous female showed a worse performance compared to wt controls, as demonstrated by the longer latency to the first nose poke in the virtual target hole (primary latency) ($p < 0.05$ after *post hoc* comparisons on the genotype by treatment interaction: $F_{(1,37)} = 7.89$; $p = 0.008$; **Figure 4A**) and the higher number of primary errors RTT mice performed ($p < 0.05$ after *post hoc* comparisons on the genotype by treatment interaction: $F_{(1,37)} = 4.41$; $p = 0.043$; **Figure 4B** (Moretti et al., 2006). Importantly, LP-211 was able to reverse this impairment and restored wt-like levels of both parameters ($p < 0.05$; **Figure 4**).

An increase in both parameters, suggestive of a worse performance in this cognitive test, was also evident in wt mice treated with LP-211 compared to veh-treated controls ($p < 0.05$). Interestingly, during both probe tests all the experimental groups showed a comparable profile. Consistently, the RM-ANOVA did not yield significant effects of repeated measurements (Probe 1 and Probe 2), or genotype by RM or treatment by RM interactions.

Electrophysiology Experiments

LTP was induced in CA1 area by TBS delivered to the Schaffer collaterals in hippocampal slices from wt and MeCP2-308 female mice, treated with either LP-211 or vehicle. TBS resulted in a long-lasting increase of fEPSP slope both in wt and in MeCP2-308 female mice (**Figure 5**). The RM-ANOVA revealed a significant main effect of RM ($F_{(48,912)} = 8.14$; $p > 0.001$) and a significant genotype by treatment interaction ($F_{(1,19)} = 6.74$; $p = 0.018$). *Post hoc* comparisons by Tukey's test on the genotype by treatment interaction showed a trend towards a reduction in the synaptic potentiation after TBS in vehicle-treated MeCP2-308 females with respect to vehicle-treated wt (**Figure 5**). A clear effect of the *in vivo* treatment was observed in RTT mice: LP-211 induced a significant increase in synaptic potentiation with respect to vehicle-treated MeCP2-308 females ($p < 0.05$; **Figure 5**), while the same treatment did not modify the degree of LTP in wt mice.

Molecular Pathways Related to Protein Synthesis

To determine whether the molecular modifications we detected at the end of a seven-day long treatment with LP-211 in the brain of RTT mice persist (De Filippis et al., 2014a), the amount and activation of rp-S6 were analyzed by western blot analyses in the hippocampi collected after a washout period of 2 months from the last (7th) ip injection of either LP-211 or vehicle. The ANOVA did not yield either genotype or treatment effects as for total rpS6 protein level (**Figure 6**). Phosphorylation levels of the rpS6 was generally reduced in the hippocampus of MeCP2-308 female mice (**Figure 6**), thus confirming the profile reported in MeCP2-mutated males (Ricciardi et al., 2011; De Filippis et al., 2014a). Such effect was however more marked at Ser240/244,

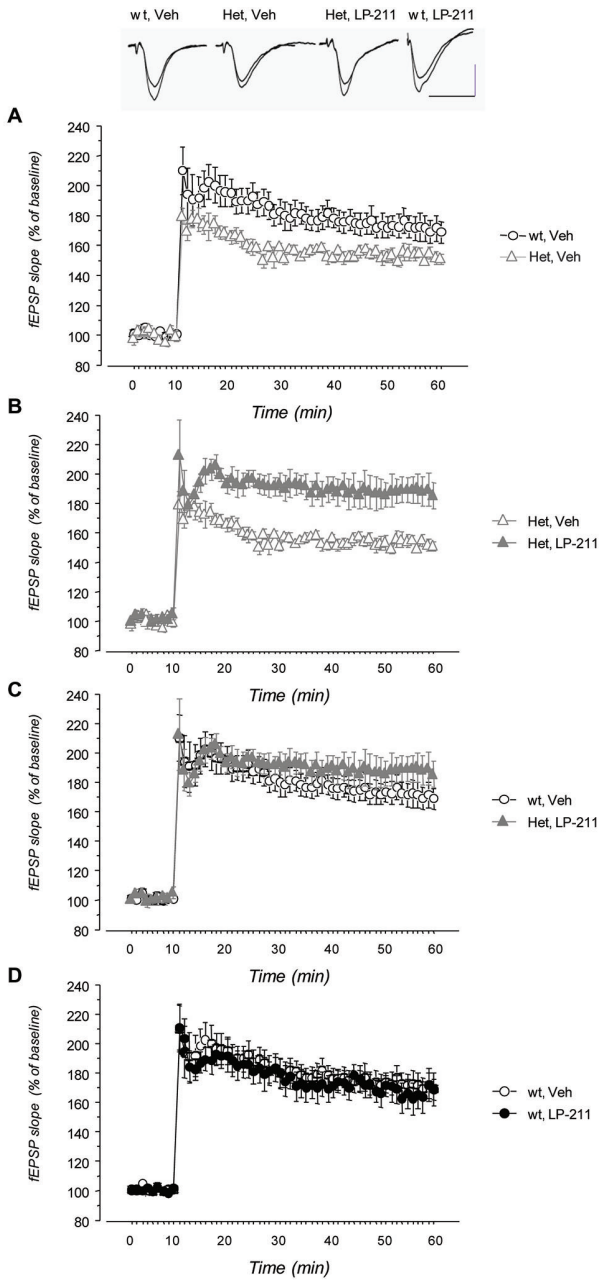

FIGURE 5 | Treatment with LP-211 improves long-term potentiation (LTP) in MeCP2-308 female mice. Field EPSPs (fEPSPs) were recorded in the CA1 area of hippocampal slices; LTP was induced by theta burst stimulation (TBS) of Schaffer collaterals. **(A)** MeCP2-308 female mice (Het, Veh) showed a reduced LTP compared to wt littermate controls (wt, Veh). **(B)** The LP-211 treatment significantly increased the response to TBS in MeCP2-308 mice (Het, LP-211) with respect to Het controls. **(C)** Comparison of the time courses of fEPSP slope after TBS did not show significant differences between LP-211-treated Het and wt mice, thus confirming a rescue of LTP in Het mice treated with LP-211. **(D)** LP-211 treatment did not affect the response to TBS in wt mice (wt, LP-211). Data are mean ± S.E.M. The number of slices for each condition was as follows: wt, Veh = 6; wt, LP-211 = 7; Het, Veh = 5; Het, LP-211 = 5. The inset shows representative fEPSPs recorded before TBS and 40 min after TBS. Each trace is the average

(*Continued*)

FIGURE 5 | Continued
of three successive fEPSPs (artifacts of stimulation have been truncated). Calibration bars: 0.5 mV, 10 ms.

than at Ser235/236 (main effect of genotype: p-rpS6 (235/236): $F_{(1,12)} = 3.86$; $p = 0.073$; p-rpS6 (240/244): $F_{(1,12)} = 238.03$; $p < 0.001$; **Figure 6**).

Consistently, the ratio between the phosphorylated forms and the total rpS6 protein content, which provides an index of the net functionality of the kinase, was shifted toward decreased phosphorylation (inactivation) in RTT mouse hippocampus compared to wt controls (main effect of genotype: Total p-rpS6/rpS6: $F_{(1,12)} = 12.75$; $p < 0.001$). Of note, LP-211 treatment significantly increased the levels of S6 phosphorylation in both genotypes (main effect of treatment: p-rpS6 (236/238)/rpS6: $F_{(1,12)} = 46.84$; $p < 0.001$; p-rpS6 (240/244)/rpS6: $F_{(1,12)} = 32.45$; $p < 0.001$) and the total p-rpS6/ rpS6 ratio (main effect of treatment: Total p-rpS6/rpS6: $F_{(1,12)} = 8.99$; $p = 0.011$), thus restoring wt-like levels of S6 phosphorylation in RTT mouse brain (**Figure 6**).

Discussion

The present study extends our previous findings (De Filippis et al., 2014a) and demonstrates that a seven-day-long stimulation of the 5-HT7 receptor significantly improves RTT-related impairments in spatial reference memory and synaptic plasticity, observed in symptomatic female mice modeling RTT. In addition, a delayed beneficial effect of the LP-211 treatment on general health status was also discovered in MeCP2-308 Het female mice. Notably, treatment evaluation on behavioral and molecular parameters was carried out up to 2 months after the last injection. The present study thus provides evidence of long-lasting beneficial effects of a transient LP-211 exposure on RTT-related impairments.

Consistent with previous studies in males (Moretti et al., 2006; De Filippis et al., 2010), we found that MeCP2-308 female mice show synaptic plasticity deficits, cognitive and motor coordination impairment as well as slight, but reliable phenotypic alterations. The present results in females confirm that neurobehavioral alterations are detectable under conditions of heterozygosis in RTT mouse models (Katz et al., 2012; Samaco et al., 2013). As the estrous cycle was not controlled in this study, we cannot completely exclude the possibility that cycle genotype differences may have exerted a role, although this seems unlikely (see the review paper: Prendergast et al., 2014). Of note, all the alterations we uncovered in RTT female mice were counteracted by a seven-day long treatment with LP-211. These include the phenotypic alterations, the impairments in cognitive and synaptic plasticity, as well as motor coordination deficits. Taken together with our previous study (De Filippis et al., 2012), these results provide evidence that a LP-211 treatment exerts a widespread beneficial effect on RTT-related symptomatology in a mouse model. Moreover, our data demonstrate that the agonist administration is equally effective when tested in the gender and the hormonal milieus which are more relevant for RTT

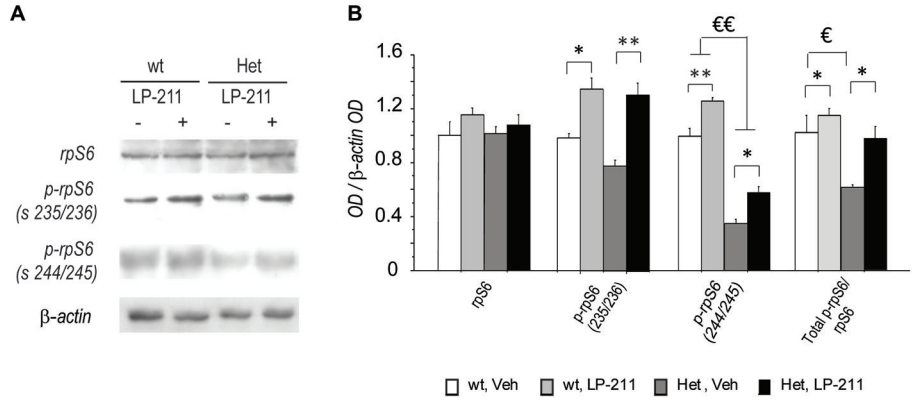

FIGURE 6 | Long-lasting beneficial effects of the stimulation of the 5-HT7 receptor on RTT-related alterations in rpS6 activity in mouse hippocampus. (A) Representative Western blot analysis (summarized view corresponding to one animal per group) of rpS6, p-rpS6 Ser 235/236, p-rpS6 Ser 240/244 and β-actin proteins in hippocampi of MeCP2-308 female mice (Het) and wt mice in control conditions (Veh) or treated with LP-211. In order to limit background and unspecific signals, the membranes related to rpS6 and p-rpS6 Ser 240/244 were cut at the opportune kDa range with the help of the MW marker before hybridization. **(B)** Semi-quantitative densitometric analysis, obtained by optical density (OD) of rpS6 signals normalized with OD of β-actin signals. OD ratios are expressed as the average fold increase vs. wt controls. $N = 4$ per each experimental groups. Data are mean ± SEM. €: wt, Veh vs. Het, Veh; $p < 0.05$; *: Veh vs. LP-211, $p < 0.05$; €€: wt, Veh vs. Het, Veh; $p < 0.01$; **: Veh vs. LP-211, $p < 0.01$.

(Katz et al., 2012), certainly increasing the translational value of the present study.

Our results provide further evidence that stimulation of 5-HT7 receptors acts consistently onto spatial memory and synaptic plasticity (Roberts and Hedlund, 2012; Volpicelli et al., 2014). As a whole, these data add to a complex picture in which 5-HT7 receptor agonists and antagonists have been reported to have both promnesic and/or anti-amnesic effects (Meneses et al., 2015), with major results pointing to an involvement of the 5-HT7 receptor in spatial memory (Hedlund, 2009; Sarkisyan and Hedlund, 2009; Beaudet et al., 2015). Interestingly, we found that the effects of the LP-211 treatment strongly depend on the basal level of performance, thus confirming previous studies (Meneses et al., 2015). In the Barnes Maze test, for instance, we found that the LP-211 treatment improved the defective performance of RTT mutants, while dampening the spatial memory of normal mice. This feature is certainly of high relevance in a translational context, in which a normalization of abnormal performances represents the most attractive outcome.

Interestingly, the beneficial effects of the treatment over the general health status of RTT mice became evident 3 weeks after the last administration of LP-211, possibly as a result of stimulated neuroplasticity. In other words, some weeks may be needed before the consequences of 5-HT7R stimulation appear, in the form of a general improvement of RTT-related neurological alterations. In particular, the phenotypic parameters which appeared to be improved in treated RTT mice include the fur, the abnormal gait, the kyphosis and the breathing abnormalities. Further studies are however needed to verify whether such effect results from an improvement in those autonomic functions which are related to aberrant serotonergic signaling in RTT, such as the respiratory brainstem dysfunction (Abdala et al., 2010, 2014). Plethysmograpic analyses will certainly help shedding light on this possibility and to corroborate our general health scoring.

By contrast, a seven-day-long treatment with LP-211 appeared to provoke a transient worsening of the general condition of wt mice and of their performance in the *Dowel test* and the *Barnes Maze task*. Phenotypic features which appeared most compromised in LP-211-treated wt mice include mobility and fur. Interestingly, we have previously reported that subchronic treatment with LP-211 reduces the 5-HT7R expression in wt mouse hippocampus, to levels comparable to those found in RTT mouse brain (De Filippis et al., 2014a). Note however that present wt mice were aged 1 year, and that a comparable treatment during adolescent age resulted in a potentiation of forebrain connectivity to the hippocampus, of 5-HT7R function in the septum as well as of spatial memory skills (Altabella et al., 2014; Canese et al., 2015). It will be interesting to determine whether the direction of LP-211 effects may depend on age, and whether modulated expression of the 5-HT7R in rodent brains may account for the phenotypic effects of a subchronic LP-211 treatment.

One major conclusion of our study concerns the enduring sequelae exerted by a seven-day-long treatment with LP-211 in RTT mice. Even though further studies are needed to uncover the neurobiological mechanisms underlying such effects, we argue that they may have been mediated by LP-211-induced activation of Rho GTPases, a family of proteins crucially involved in intellectual disability disorders (Ramakers, 2002; De Filippis et al., 2014b). Our previous data do in fact demonstrate that the effects of a single intra-cerebro-ventricular injection with CNF1, a bacterial protein known to transiently activate Rho GTPases, were still well evident after months in a RTT mouse model (De Filippis et al., 2012). In the same study, we unequivocally demonstrated the crucial role played by Rho GTPases: the abolition of the CNF1 activity over the activation status of Rho

GTPases was sufficient to prevent the beneficial effects of this focal CNF1 treatment.

Rho GTPases are a family of proteins which plays a crucial role in the regulation of synaptic plasticity, synaptogenesis and dendritic spine formation (Luo, 2000; Etienne-Manneville and Hall, 2002; Hall, 2005). Hence, it may be speculated that plastic remodeling of the neural circuitries might account, in RTT mouse brains, for the apparent long-lasting effects of pharmacological approaches targeting Rho GTPases, like CNF1 and LP-211 (Diana et al., 2007; Cerri et al., 2011; De Filippis et al., 2012; Loizzo et al., 2013). In this line, we have recently demonstrated that a sub-chronic treatment with LP-211 during adolescence, a time window of increased plasticity of the central nervous system (Adriani et al., 2006), induces a persistent rearrangement of neural circuitries in rats (Altabella et al., 2014). A similar neuroplastic effect might thus account for the long-lasting beneficial effects of LP-211 we presently report in RTT mice. Further studies are however needed to clarify this point.

Notably, we found that the molecular effects of LP-211 treatment over the activation status of the rpS6, the downstream target of mTOR and S6 kinase responsible for the altered protein translational control in RTT mouse brain (Ricciardi et al., 2011), were still well evident after 2 months from the last injection of LP-211 in RTT mouse hippocampus. Even though the relative role of these molecular effects at the phenotypic level is still unclear, such results are particularly interesting as the normalization of this signaling pathway in an *in vitro* RTT human model is known to rescue disease-related cellular impairments (Li et al., 2013). We cannot at the moment explain how this persistent molecular effect is achieved, and further studies are needed. We can, however, certainly exclude that it can be related to brain accumulation of LP-211 or to persistent binding to the 5-HT7R, based on previous studies demonstrating that LP-211 is no longer detectable in mouse brain after 120 min from a single (10 mg/kg, ip) injection (Leopoldo et al., 2008).

Conclusion

Overall, the present study extends previous findings from our laboratory, also highlighting persistent effects, and provides compelling preclinical evidence of the potential therapeutic value of a pharmacological approach targeting the brain serotonin receptor 7. This innovative approach may turn out to be relevant for RTT, a devastating disorder for which no cure is currently available. The high potential of innovative drugs, capable of targeting the activation status of the Rho GTPases family, is also further supported. Given the promising results so far obtained at the preclinical level, any effort to narrow the gap between preclinical and clinical research is now urgent and mandatory.

Author Contributions

Design of the work: BDF, MRD, AF, GL; Synthesis of LP-211: EL, ML; Data acquisition and analysis: BDF, VC, MRD, AF; Interpretation of data: All authors; Manuscript preparation: BDF; Revision of the manuscript: All authors.

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5-HT$_7$ receptor signaling: improved therapeutic strategy in gut disorders

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Serotonin (5-hydroxytryptamine; 5-HT) is most commonly known for its role as a neurotransmitter in the central nervous system (CNS). However, the majority of the body's 5-HT is produced in the gut by enterochromaffin (EC) cells. Alterations in 5-HT signaling have been associated with various gut disorders including inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and enteric infections. Recently, our studies have identified a key role for 5-HT in the pathogenesis of experimental colitis. 5-HT$_7$ receptors are expressed in the gut and very recently, we have shown evidence of 5-HT$_7$ receptor expression on intestinal immune cells and demonstrated a key role for 5-HT$_7$ receptors in generation of experimental colitis. This review summarizes the key findings of these studies and provides a comprehensive overview of our current knowledge of the 5-HT$_7$ receptor in terms of its pathophysiological relevance and therapeutic potential in intestinal inflammatory conditions, such as IBD.

Keywords: inflammatory bowel disease, serotonin receptor type 7

INTRODUCTION

The gastrointestinal (GI) tract contains an extensive system of endocrine cells that are interspersed amongst gut epithelial cells (Rehfeld, 1998). There are several subpopulations of endocrine cells, which release various biologically active compounds such as gastrin, secretin, cholecystokinin, chromogranin, and serotonin (5-hydroxytryptamine; 5-HT; Sharkey and Mawe, 2002). Enterochromaffin (EC) cells are the best characterized subset of enteric endocrine cells and constitute the largest endocrine cell population in the gut (Ham, 2002; Sharkey and Mawe, 2002; Ku et al., 2006; Gunawardene et al., 2011). EC cells arise from multipotent stem cells located near the base of the crypts of Lieberkühn and are the body's main source of 5-HT (Sharkey and Mawe, 2002; Gunawardene et al., 2011). Alterations in EC cells and 5-HT signaling has been shown to be associated in a number of GI disorders including inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and enteric infections, emphasizing the importance of 5-HT signaling in intestinal homeostasis.

5-HT mediates many GI functions, including secretion and peristalsis, by activating of a diverse range of 5-HT receptors (Mawe and Hoffman, 2013). To date, seven types of 5-HT receptors have been identified and amongst these, five are expressed within the GI tract. This paper reviews information on the most recently identified class of 5-HT receptors, 5-HT$_7$, and its role in the GI tract with a focus on its implications in understanding the pathophysiology of intestinal disorders.

5-HT IN THE GI TRACT

5-HT is a highly conserved biogenic amine that is found in separate peripheral and central tissue pools that are distinctly regulated by two different rate-limiting enzymes, tryptophan hydroxylase (TpH) 1 and 2, respectively (Walther and Bader, 2003; Walther et al., 2003). TpH1 found in EC cells catalyzes the majority of 5-HT production in the body (roughly ∼90%). TpH1 converts dietary L-tryptophan to 5-hydroxytryptophan (5-HTP), which is then converted to 5-HT by L-amino acid decarboxylase (Bertrand and Bertrand, 2010). Once synthesized, 5-HT is packaged into granules by the vesicular monoamine transporter 1 (VMAT1; Rindi et al., 2004; Schäfermeyer et al., 2004) and released mainly from granules stored near the basal border of the EC cell, though some studies have identified granules near the apical membrane (Nilsson et al., 1987). EC cells release 5-HT in response to various mechanical and chemical stimuli, including bacterial toxins, in a calcium-dependent manner (Racké et al., 1996; Mössner and Lesch, 1998). Once released, 5-HT participates in various gut functions, including secretion and peristalsis, by activation of a diverse range of 5-HT receptors located in the lamina propria (LP; Mawe and Hoffman, 2013). The actions of 5-HT are terminated by uptake by the serotonin reuptake transporter (SERT) into adjacent epithelial cells (Martel et al., 2003) and degradation by monoamine oxidase A (MAO_A). SERT is also found in platelets and enteric neurons (Bertrand and Bertrand, 2010), and is the target for important

therapeutic drugs such as fluoxetine and citalopram, members of the family of serotonin-selective reuptake inhibitors (SSRIs). SERT mediated reuptake of 5-HT can be partially replaced by the dopamine transporter (DAT) and organic cation transporter (OCT), albeit at a lower affinity than SERT (Chen et al., 2001).

5-HT SIGNALING IN INTESTINAL INFLAMMATION

IBDs, Crohn's disease (CD) and ulcerative colitis (UC), are serious chronic inflammatory conditions of the human bowel currently affecting approximately 1–2 million people in the US and Canada (Loftus, 2004). Although the pathogenesis of IBD remains unknown, it is a multifactorial disease that involves both genetic and environmental components. As such, IBD is considered to be an inappropriate immune response that occurs in genetically susceptible individuals as a result of a complex interaction between environmental factors, microbial factors, and the intestinal immune system (Bouma and Strober, 2003; Bernstein and Shanahan, 2008; Koloski et al., 2008; Arnett and Viney, 2010). During the past five decades, the frequency of IBD has increased rapidly in highly industrialized Western nations with Canada having one of the highest incidence rates of both UC and CD worldwide (Fedorak et al., 2010).

Alterations in 5-HT signaling have been observed in IBD (Ahonen et al., 1976; Belai et al., 1997; El-Salhy et al., 1997; Magro et al., 2002; Coates et al., 2004) and changes to EC cell numbers and 5-HT content have been associated with both UC and CD (Bishop et al., 1987; Belai et al., 1997; El-Salhy et al., 1997). Changes in 5-HT signaling have also been shown in various experimental models of intestinal inflammation, including trinitrobenzene sulphonic acid (TNBS), di-nitrobenzene sulphonic acid (DNBS), and dextran sulfate sodium (DSS; Oshima et al., 1999; Linden et al., 2005; Khan et al., 2006). In all of these models, EC cell numbers and 5-HT levels are increased. In addition, infection with either *Trichuris muris* or *Citrobacter rodentium*, leads to an increase in EC cells numbers and/or 5-HT release, further supporting a role for 5-HT in inflammatory states (O'Hara et al., 2006; Motomura et al., 2008).

5-HT itself also plays a key role in the generation of intestinal inflammation. Previously, we have shown that there is significant reduction in intestinal inflammation post- DSS and DNBS-induced colitis when intestinal 5-HT levels are reduced by genetic deletion of the rate-limiting TpH1 enzyme or by using parachlorophenylalanine (pCPA), while replenishing 5-HT levels intensifies colitis severity (Ghia et al., 2009). In turn, studies have also shown that chemical-induced colitis or spontaneous colitis associated with an IL-10 deficiency is increased in severity when coupled with the 5-HT enhancing effects of a knockout of SERT (Bischoff et al., 2009; Haub et al., 2010). Prior approaches aimed at blocking 5-HT synthesis by a pharmacological agent through inhibition of TpH, as with pCPA, have been impeded by adverse effects to brain 5-HT synthesis leading to alterations in central nervous system (CNS)-mediated functions (Ruhé et al., 2007). Recently, we have also shown that blocking 5-HT synthesis using an orally-delivered small molecule TpH inhibitor, telotristat etiprate (LX1032/LX1606), effectively reduces peripheral 5-HT synthesis and both chemical- and infection-induced intestinal inflammation (Kim et al., 2013b). This compound is unable to cross the blood-brain barrier (Savelieva et al., 2008) and does not appear to affect enteric neuronal TpH2 (Margolis et al., 2013). Oral administration of LX1606 significantly depletes intestinal 5-HT levels but does not affect brain 5-HT levels. Margolis et al. (2013) also evaluated the effect of LX1606 on neuronal 5-HT stores using immunocytochemical techniques and found that LX1606 did not affect the proportion of myenteric 5-HT-immunoreactive neurons or the area of myenteric plexus occupied by 5-HT-immunoreactive nerve fibers. This suggests that while LX1606 significantly depletes 5-HT stores from EC cells, entire neuronal 5-HT stores are maintained and therefore, LX1606 and similarly related peripheral TpH inhibitors appear to fail to enter the myenteric plexus and/or inhibit enteric neuronal TpH2.

The precise mechanisms by which 5-HT exerts its pro-inflammatory actions remains to be determined. To elucidate this mechanism, we assessed the role of 5-HT in dendritic cell (DC) function in relation to gut inflammation. EC cells are located in very close proximity to or in contact with immune cells such as DCs (Yang and Lackner, 2004) and studies from our lab and others have shown an important role for 5-HT in immune regulation and in turn, immune-mediated alteration of EC cells/5-HT signaling (Wang et al., 2007; Li et al., 2011; Shajib et al., 2013). DCs are professional antigen-presenting cells with the ability to initiate adaptive immune responses. Intestinal DCs reside in the LP as such, are able to continuously sample luminal contents. DCs play a critical role in orchestrating immune responses and have been shown to be important in the generation of intestinal inflammation (Lipscomb and Masten, 2002; Berndt et al., 2007). DCs isolated from TpH1 deficient mice following DSS administration release significantly less IL-12 compared with DCs isolated from wild-type mice (Li et al., 2011). Interestingly, when DCs isolated from TpH1 deficient mice are cultured in the presence of 5-HT, this restores IL-12 levels to those comparable to DCs from wild-type mice suggesting a role of 5-HT mediated activation of DCs. Furthermore, when 5-HT stimulated DCs are transferred back into TpH1 deficient mice, there is significant increase in colitis severity and this is associated with higher myeloperoxidase (MPO) activity and pro-inflammatory cytokine (IL-1β and IL-6) levels. This suggests that 5-HT mediated modulation of DC function is important in the pathogenesis of colitis though research targeting 5-HT signaling is needed to translate these observations for clinical utilization and to design a therapeutic strategy for colitis.

INTESTINAL INFLAMMATION AND THE GUT-BRAIN-AXIS

The gut-brain-axis is a bi-directional neuro-humoral communication system that links gut and brain function in health and disease, and contributes to GI functions, including motility, secretion, visceral sensations, and mucosal immunity (Collins and Bercik, 2009; El Aidy et al., 2012; Forsythe and Kunze, 2013). The importance of this axis is demonstrated by its role in IBS and is reflected in the high prevalence of psychiatric morbidity in IBS (Whitehead et al., 2002). There is also growing evidence that the gut-brain-axis plays a role in IBD (Graff et al., 2009; Bonaz and Bernstein, 2013). IBD results in high morbidity and mortality and severely compromises quality of life and life expectancy. In

recent years, there has been increasing recognition that depression can worsen the course of IBD (Mardini et al., 2004; Mittermaier et al., 2004; Persoons et al., 2005) and it has been shown that persons with IBD have higher rates of depression (in addition to panic, generalized anxiety, obsessive-compulsive disorders) compared with control populations (Walker et al., 2008). The mechanisms underlying this relationship in terms of cause-and-effect are currently unclear. In a study by Walker et al. (2008), it was reported that patients with IBD have a higher 12-month and also lifetime prevalence of major depression whereby approximately half experienced a first episode of depression more than 2 years before the onset of IBD. Depression may also negatively affect the course and outcome of disease. A prospective study by Mittermaier et al. (2004) found that in patients with IBD, those with significant depressive symptoms (at baseline) had relapses that occurred sooner and more frequently. In patients with CD, major depressive disorder has been reported as a risk factor for failure to achieve remission with infliximab treatment and an earlier need for retreatment (Persoons et al., 2005). In addition, studies have found that patients with active disease report higher levels of depression and anxiety while those with quiescent disease report lower levels (Porcelli et al., 1996; Levenstein, 2002; Larsson et al., 2008). As such, it has been proposed that treatments that improve mood may be useful in improving symptoms and disease activity in IBD. Antidepressants such as SSRIs are generally well tolerated and are successful in relieving psychological symptoms in about 30–40% of patients (Trivedi et al., 2006; Krishnan and Nestler, 2008). Recently, it was reported that antidepressants used to treat concomitant mood disorders in patients with IBD improves relapse rates and use of corticosteroids when compared with matched controls. Whether this occurred through a direct effect of the drug on the GI tract, or indirectly via improvement in mood and stress response was not investigated (Goodhand et al., 2012). Uncontrolled case report studies have also reported improvements not only in depression but also in IBD symptom scores (Mikocka-Walus et al., 2006). In contrast, data from a single open-label study of SSRI in IBD patients with depression reported improvements in depression but not in IBD activity (Walker et al., 1996). Moving forward, there is a need for randomized controlled trials to assess the effects of antidepressants such as SSRIs on disease activity in patients with IBD.

Studies using animal models have provided insight into mechanisms involved in the gut-brain-axis during GI inflammation and infection. Lyte et al. (1998) showed that mice treated orally with *Campylobacter jejuni* had increased anxiety-like behavior compared to saline-treated control mice. This was without any increase in inflammatory mediators and likely due to activation of vagal ascending pathways. In experimental models that result in increased GI inflammation, there are increases in anxiety-like behavior. In animal models of colitis, mice treated with DSS show increased anxiety-like behavior (Bercik et al., 2011). In addition, mice infected with *Trichuris muris* demonstrated intestinal inflammation that was associated with increased anxiety-like behavior when tested using the light/dark test and step-down test methods (Bercik et al., 2010). This was accompanied by decreased brain derived neurotropic factor (BDNF) expression in the hippocampus, and elevated levels of TNF-α, INF-γ, and kynurenine. Abnormal behavior (but not BDNF levels) was normalized by treatment with immunomodulators, etanercept and budesonide. Interestingly, both behavior and BDNF levels normalized following administration with probiotic *Bifidobacterium longum* suggesting a role for gut microbiota in modulating behavior. The role of the microbiota on the gut-brain-axis, however, is beyond the scope of this review, and has been extensively reviewed elsewhere (Cryan and O'Mahony, 2011; Collins et al., 2012). What is clear from the growing body of literature, is that the gut microbiome plays a critical role in regulating normal function of the gut-brain axis. Recently, there is a growing body of evidence looking at the role of 5-HT and the gut microbiome suggesting that 5-HT may be critically involved at every level of the brain-gut-microbiome axis (as reviewed by O'Mahony et al., 2014). With a better understanding of the interaction between this axis and the 5-HT system, this could aid in the design and development of novel therapeutic strategies for intestinal disorders that target 5-HT signaling with far-reaching effects beyond the gut. This may be particularly relevant in GI inflammatory disorders such as IBS and IBD with reported psychiatric comorbidities.

5-HT$_7$ RECEPTORS IN THE GUT

The discovery of 5-HT in the late 1940s was shortly followed by evidence for 5-HT receptor heterogeneity. To date, seven distinct families of 5-HT receptors have been identified, with some families consisting of various subpopulations (Hoyer et al., 2002). Five of the seven known families (5-HT$_1$, 5-HT$_2$, 5-HT$_3$, 5-HT$_4$, and 5-HT$_7$ receptors) are expressed in the gut (Hoyer et al., 2002), with the 5-HT$_3$ and 5-HT$_4$ receptor subtypes being the most extensively studied. 5-HT$_3$ and 5-HT$_4$ receptors have been targeted for the treatment of diarrhea and constipation, respectively (Mawe and Hoffman, 2013). The 5-HT$_7$ receptor is the most recently discovered member of the 5-HT receptor family and has since been cloned in rat (Lovenberg et al., 1993; Meyerhof et al., 1993; Ruat et al., 1993; Shen et al., 1993), mouse (Plassat et al., 1993), guinea pig (Tsou et al., 1994), porcine (Bhalla et al., 2002), and human (Bard et al., 1993).

The 5-HT$_7$ receptor is expressed in both the CNS and in peripheral tissues. In the CNS, pharmacological and animal studies using 5-HT$_7$ receptor deficient mice have established roles for the 5-HT$_7$ receptor in control of circadian rhythms and thermoregulation (Lovenberg et al., 1993; Tsou et al., 1994; Hedlund et al., 2003), learning and memory (Roberts and Hedlund, 2012), and mood disorders including depression (Hedlund, 2009; Mnie-Filali et al., 2009). In the periphery, 5-HT$_7$ receptors have been found to be expressed in the colon, ileum, and stomach with low expression in the spleen, liver, and kidney (Bard et al., 1993). 5-HT$_7$ receptors have also been reported to be expressed on human enterocyte-like cell line, Caco-2 cells, and was found to modulate SERT activity (Iceta et al., 2009). Blood-derived DCs also express the 5-HT$_7$ receptor (Shen et al., 1993; Vanhoenacker et al., 2000; Idzko et al., 2004).

Specifically within the gut, 5-HT$_7$ receptors are expressed on smooth muscle cells, enteric neurons, and within the solitary intestinal lymphoid tissue, small-sized intestinal lymphoid structures scattered through the small intestine (Tonini et al., 2005; Guseva et al., 2014). Recently, we have shown that the 5-HT$_7$

receptor is also expressed on intestinal LP DCs (Kim et al., 2013a; **Figure 1**). DCs represent a heterogeneous population with functional diversity with different DC subsets having distinct sets of cell surface antigens. Although CD11c is the classical integrin marker used to distinguish DCs from macrophages (whereby CD11b$^+$ CD11c$^-$ and CD11b+/− CD11c^{high} are classified as macrophages and DCs, respectively), this becomes more difficult when distinguishing between LP macrophage and DC populations, as LP macrophages express both CD11b and CD11c markers (Mowat and Bain, 2011). Therefore, it is important to use differential expression of integrin CD103 (αE integrin) to reliably distinguish between these two populations. We found that isolated intestinal CD103$^+$ CD11c$^+$ cells were positive for 5-HT$_7$ receptor expression whereas no significant amount was detected on CD103$^-$ CD11c$^+$ cells. Adding to this finding, Guseva et al. (2014) recently reported that CD11c+ CD86+ cells colocalize with 5-HT$_7$ receptor staining in colon samples collected from both inflamed and non-inflamed areas of patients with CD. CD86 is a co-stimulatory molecule found on mature DCs. These findings suggest that 5-HT$_7$ receptor expressed by DCs may play a role in modulating intestinal inflammation in this patient population.

Under physiological conditions, there is evidence that 5-HT$_7$ receptors play a role in motility by mediating smooth muscle relaxation in colon (Prins et al., 1999; Tonini et al., 2005) and ileum smooth muscle (Carter et al., 1995). In addition, they are believed to have a role in initiating murine colonic migrating motor complexes (Dickson et al., 2010). 5-HT$_7$ receptors may also have a role in inhibition of peristalsis by 5-HT (Tuladhar et al., 2003; **Table 1**).

5-HT$_7$ RECEPTOR SIGNALING IN INTESTINAL DISORDERS

IRRITABLE BOWEL SYNDROME

A number of studies have reported altered 5-HT signaling activity in intestinal disorders. Therapeutic drugs to target selective modulation of 5-HT activity—including SSRIs, 5-HT$_3$ and 5-HT$_4$ antagonists and agonists, respectively—have been used in the treatment of functional GI disorders such as IBS (Gershon and Tack, 2007; Beattie and Smith, 2008). Some of these drugs, however, have been associated with unwanted side effects (Ladabaum, 2003; Cole et al., 2004) and thus, prompts the need for more studies on 5-HT and its receptors in GI pathology and pathophysiology. IBS is a functional bowel disorder in which abdominal

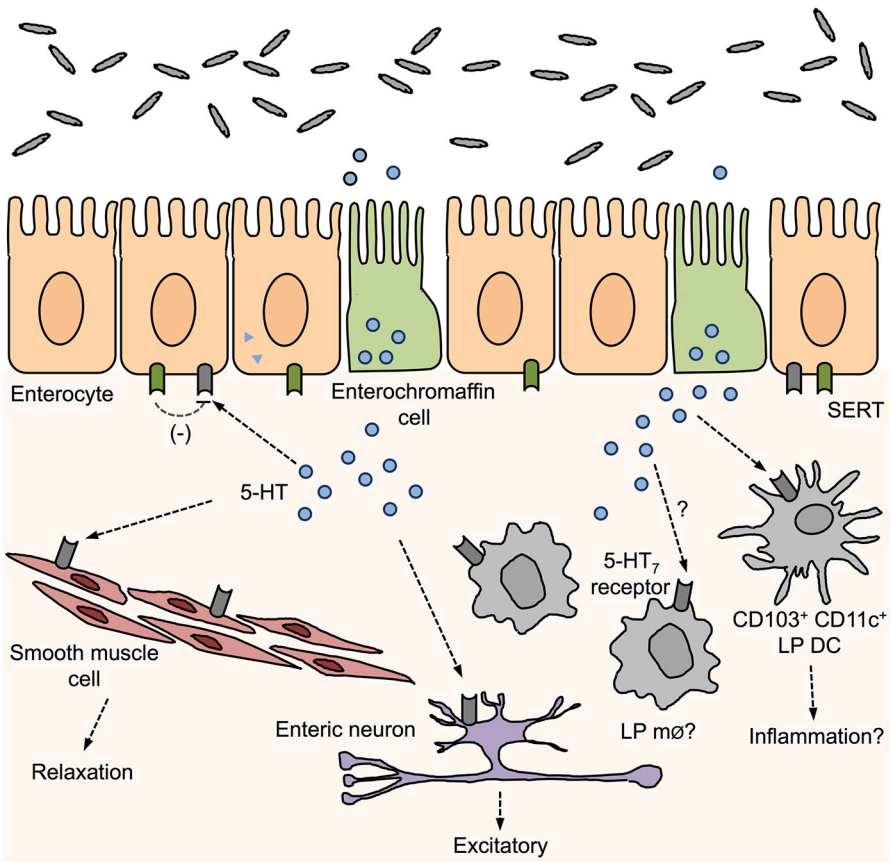

FIGURE 1 | Distribution of 5-HT$_7$ receptors in the gut and proposed roles in gut function. 5-hydroxytryptamine (5-HT) released from enterochromaffin cells can act on surrounding 5-HT$_7$ receptors that are expressed by smooth muscle cells, enteric neurons, enterocytes, and immune cells. Activation of 5-HT$_7$ receptors can influence muscle tone, enteric neuron excitation, and have been proposed to inhibit SERT activity and promote inflammation by activation of LP dendritic cells (DCs).

Table 1 | Effects of 5-HT$_7$ receptor activation or inactivation on gut function.

Species/Region	5-HT$_7$ activation/inhibition	Effect	Reference
Guinea pig ileum	Antagonist SB-269970	Restores 5-HT inhibited peristalsis	Tuladhar et al. (2003)
	Antagonist SB-269970	Inhibits 5-HT inhibited peristalsis (via enteric neurons)	Tonini et al. (2005)
	Agonist 8-OH-DPAT	Elicits relaxation	Carter et al. (1995)
Guinea pig mesentary	Antagonist SB-269970	Inhibits 5-HT inhibited constriction	Chan and von der Weid (2003)
Mouse colon	Antagonist SB-269970	Inhibits spontaneous	Dickson et al. (2010)
	Antagonist SB-258719	colonic migrating motor complexes	
Canine stomach	Antagonist SB-269970	Inhibits 5-Carboxamidotryptamine (5-CT) induced gastric relaxation	Janssen et al. (2002)
Human colon	Antagonist SB-269970	Inhibits 5-HT induced relaxation of human colonic circular muscle	Irving et al. (2007)
	Antagonist mesulergine	Inhibits 5-HT induced relaxation of circular muscle	Prins et al. (1999)

pain and discomfort is associated with altered bowel habits. 5-HT plays a key role in regulating motor functions of the GI tract and studies have suggested that 5-HT$_7$ receptors mediate smooth muscle relaxation, adding to the rationale for investigating 5-HT$_7$ receptor ligands in IBS. In addition, they play a role in regulation of nociceptive pathways (Meuser et al., 2002) and thus, may be involved in the pathological mechanisms underlying visceral paresthesia seen in IBS. Zou et al. (2007) investigated the role of 5-HT$_7$ receptors in the pathogenesis of IBS in a rodent model and found that 5-HT$_7$ receptor expression was increased in the hippocampus, hypothalamus, and intestine (ileum and colon) of IBS groups as compared to controls and this was associated with higher cAMP levels at these sites. In addition, there is a high prevalence of comorbid depression and anxiety disorders in IBS patients (Andresen and Camilleri, 2006). As 5-HT$_7$ receptors have been linked with depression (although its role in anxiety is currently inconclusive) (as reviewed by Hedlund, 2009), it may be an attractive potential therapeutic target for IBS with effects extending beyond the gut.

INFLAMMATORY BOWEL DISEASE

The role of 5-HT$_7$ receptors in IBD is far less studied. Very recently, however, Guseva et al. (2014) has reported that 5-HT$_7$ receptor expression is increased in inflamed sections of CD patients. In addition, using experimental models of colitis, we have previously reported that 5-HT$_7$ receptor levels are increased in the colon of mice post- DSS-induced colitis (Kim et al., 2013a). In addition, Guseva et al. (2014) also found an up-regulation in 5-HT$_7$ receptor expression in cecum and rectum in DSS-treated animals compared with controls. Ours and other studies have also shown that the 5-HT$_7$ receptor is expressed on DCs (Idzko et al., 2004; Kim et al., 2013a; Guseva et al., 2014). EC cells, the major producer of 5-HT in the gut, are located in close proximity to these cells and thus, it is likely that there is interplay between these two systems. We proposed that inhibiting 5-HT signaling by blocking 5-HT$_7$ receptor function would lead to attenuation of immune cell activation and subsequent inflammation. Indeed, we found that blocking 5-HT signaling by using a selective 5-HT$_7$ receptor antagonist (SB-269970) or by genetic deletion of this receptor alleviated intestinal inflammation in two separate chemical models of colitis (DSS and DNBS) (Kim et al., 2013a). This was indicated by lower macroscopic damage of the colon and less severe histopathological damage as compared to vehicle-treated controls. In turn, this was associated with a decrease in various pro-inflammatory markers including MPO and cytokines IL-1β, IL-6, and TNF-α. The beneficial effects of targeting said receptor was also seen in a chronic model of DSS-induced colitis. Furthermore, we identified a role for 5-HT$_7$ receptor mediated cytokine release by mature DCs isolated from DSS-treated mice. Similar effects of 5-HT on cytokine secretion have been observed in monocyte-derived DCs (Müller et al., 2009). Importantly, it was recently reported that increased 5-HT$_7$ receptor-positive cells in DSS-treated mice were also positive for CD11c, suggesting that there is increased amount of 5-HT$_7$ receptor expressing DCs upon inflammation (Guseva et al., 2014).

By using chimeric mice that were reconstituted with bone marrow (BM) cells lacking 5-HT$_7$ receptor expression, we found that wild-type recipients that received BM cells from 5-HT$_7$ receptor deficient donors showed lower disease activity and less severe histopathological damage (Kim et al., 2013a). This suggests that 5-HT$_7$ receptor activation on immune cells play a key role in mediating intestinal inflammation in experimental colitis. Contrary to our data, Guseva et al. (2014) found that pharmacological blockade or genetic deletion of the 5-HT$_7$ receptor in DSS-induced colitis exacerbated colitis severity in mice. This may in large be due to the notable differences in experimental design and housing condition of animals. For instance, Guseva et al. (2014) administered the 5-HT$_7$ receptor antagonist at a lower dosage (100 nM) for a shorter period of time, while we administered the antagonist throughout the duration of the DSS treatment at a much higher dosing range (20–80 mg/kg).

DCs are important players that orchestrate downstream immune responses from initial sampling of the luminal environment. As such, DCs play a crucial role in priming responses to Th1 and Th17 cells (important producers of IFN-γ and IL-17, respectively). We reported alterations in colonic IFN-γ and IL-17 levels in 5-HT$_7$ receptor antagonist-treated mice post DSS-induced colitis suggesting that there may be effects on T-cell responses when 5-HT$_7$ receptor signaling is disrupted *in vivo* (Kim et al., 2013a). Further investigation of the role of 5-HT$_7$ receptor signaling in T-cell function and examination of the role of DCs and sequential T cell activation in

the context of gut inflammation will be necessary in order to elucidate the downstream effects of targeting this receptor in intestinal inflammation. In addition, the role of 5-HT$_7$ receptor activation in modulating other DC functions such as immune cell survival, remain to be determined. Recently, it has been reported that 5-HT stimulates human macrophage polarization through 5-HT$_{2B}$ and 5-HT$_7$ receptors pointing to 5-HT as a potential target for modulating macrophage polarization and the potential of targeting 5-HT$_{2B}$ and 5-HT$_7$ receptors as therapies against inflammatory pathologies (de las Casas-Engel et al., 2013). Future studies investigating the role of these receptors on LP macrophages in the context of colitis would be interesting to determine the specific roles the 5-HT$_7$ receptor has on different cell types and subpopulations. Given the heterogeneity seen amongst immune cells in the intestine, it would not be surprising for 5-HT$_7$ receptor activation to play distinct roles in different cell types.

ENTERIC INFECTIONS

Alteration in the number of EC cells and 5-HT levels have been observed in enteric infection (Bearcroft et al., 1996; Spiller et al., 2000; Turvill et al., 2000; Kordasti et al., 2004; Wheatcroft et al., 2005). Recently, we examined the role of the 5-HT$_7$ receptor during enteric infection by using a murine model of large intestinal nematode infection, *Trichuris muris*. We found that expulsion of worms was significantly delayed in 5-HT$_7$ receptor deficient mice after *T. muris* infection and this was accompanied by an attenuation of infection-induced colonic muscle contractility. There was reduction in IL-9 levels in 5-HT$_7$ receptor deficient mice as compared to wild-type after infection (Kim et al., 2012). This suggests that the 5-HT$_7$ receptor plays an important role in generation of infection-induced intestinal muscle contractility, worm expulsion, and modulation of immune responses in the context of host defense in enteric infection.

CONCLUSIONS AND FUTURE DIRECTIONS

There is now abundant evidence in favor of an important role of 5-HT signaling in various gut disorders including IBD, IBS, and enteric infections. In the past two decades, there have been significant advances in our understanding of the 5-HT$_7$ receptor. Despite our best efforts, there are many unanswered questions and new avenues of research that still warrant investigation. Here, we highlighted the role of this receptor in the gut specifically in relation to inflammation and functional disorders; however, novel functions and roles for these receptors continue to emerge. Further elucidating the role of these receptors in intestinal function, and in intestinal pathologies and pathophysiology will help us to better understand the underlying mechanisms of various common intestinal disorders such as IBD and IBS, and will ultimately lead to the development of novel therapies. Further studies investigating 5-HT$_7$ receptor signaling in human samples from IBD patients and targeted inhibition of 5-HT$_7$ receptor function on mucosal DCs will be important in determining the role of the 5-HT$_7$ receptor in intestinal inflammation and to translate *in vivo* findings.

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