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# Involvement of the serotonin receptor 7 in synaptic plasticity in the nervous system


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Это - факт.

А факт - самая упрямая в мире вещь.

This is a fact.

And a fact is the most stubborn thing in the world.

(The Master and Margarita- M.Bulgakov)

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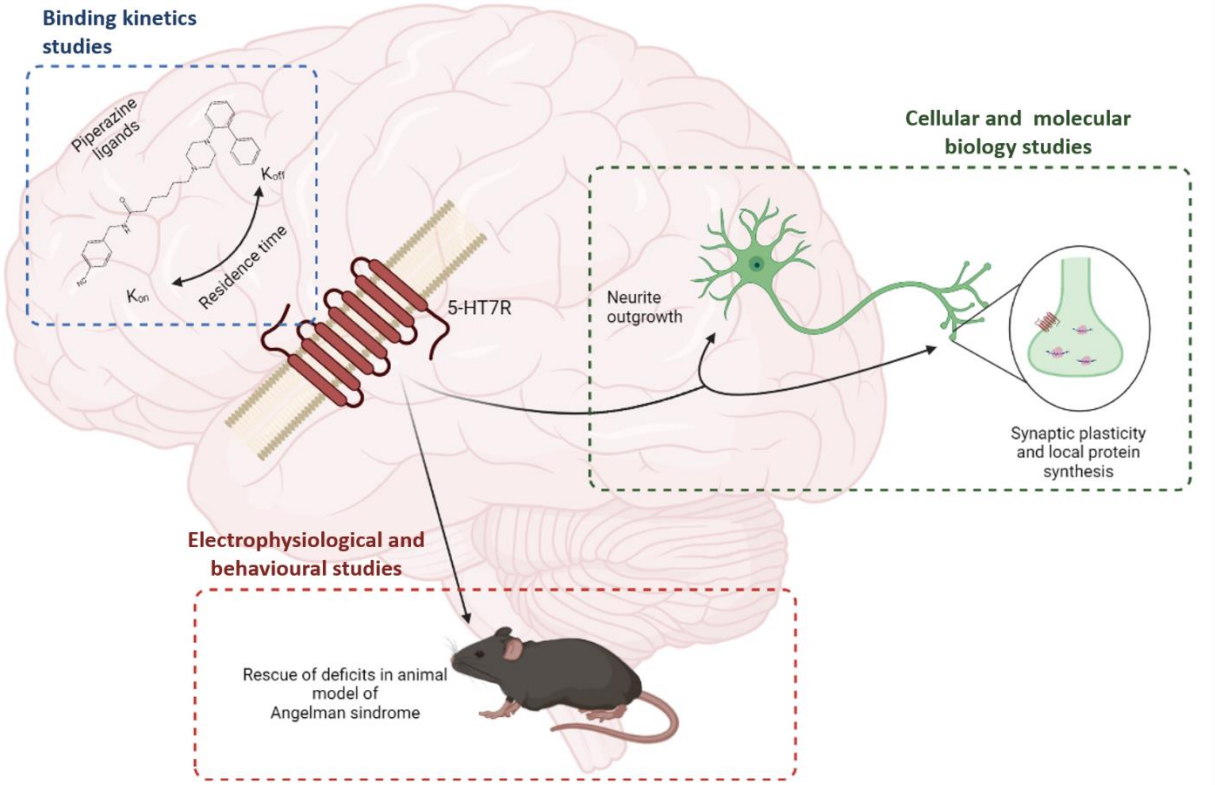
# 1. GRAPHICAL ABSTRACT



# 2. INTRODUCTION

## 2.1 Serotonin and serotonergic neurons

Serotonin (5-hydroxytryptamine or 5-HT), a phylogenetically ancient signaling molecule (Hay-Schmidt, 2000), is the most widely distributed neurotransmitter in the brain (Steinbusch, 1981). It was first discovered in the gut and enterochromaffin cells and subsequently detected in blood serum as a vasoconstrictor, hence the name serotonin (*serum that gives tone*; Rapport et al., 1948). Indeed, 5-HT plays a key role not only in brain, but also in peripheral tissues. 5-HT is synthesized from the amino acid tryptophan, which is hydroxylated to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (TPH). 5-HTP is converted into 5-HT by aromatic L-amino acid decarboxylase. The enzyme TPH is the rate limiting enzyme for 5-HT biosynthesis and has two distinct isoforms encoded by two genes: *Tph1* expressed in peripheral tissues and pineal gland, and *Tph2* selectively expressed in the Central Nervous System (CNS) and in the enteric neurons of the gut (Pratelli and Pasqualetti 2019). Studies on rodent models confirmed that 5-HT synthesis in the brain is driven by TPH2, whereas 5-HT synthesis in peripheral organs is driven by TPH1 (Mosienko et al 2019). Since 5-HT is unable to cross the blood–brain barrier, at least in adult, the central and the peripheral serotonergic systems are independently regulated. Synaptic 5-HT concentrations and signaling are mainly regulated by the 5-HT transporter (SERT), which transfers 5-HT from the synaptic cleft back into presynaptic terminals after its release (Rudnick and Sandtner 2019).

Interestingly, 5-HT was the first neurotransmitter for which a developmental role in the nervous system was suggested (Nakamura and Hasegawa 2003). Indeed, brain 5-HT plays a key role in modulating neuronal circuit development and activities, regulating a plethora of functions such as sleep and circadian rhythms, mood, memory and reward, emotional behaviour, nociception and

sensory processing, autonomic responses, and motor activity (Pilowsky 2019). These numerous functions are the consequence of the extensive axonal network of the serotonergic neurons, capable to reach and influence nearly all the CNS areas.

The influence of 5-HT neurotransmission in the CNS is made more complex by the interaction of the 5-HT system with many other classical neurotransmitter systems. The activation of serotonergic receptors located on cholinergic, dopaminergic, GABAergic or glutamatergic neurons, modulates the neurotransmitter release of these neurons (Seyedabadi et al 2014; De Deurwaerdère et al 2017). Moreover, 5-HT neurons may release also other classical neurotransmitters such as glutamate (Sengupta et al 2017), and other amino acids (Fernandez et al 2016), in a process defined as 'cotransmission', which is still poorly understood and the object of intense investigation (Svensson et al 2018).

### 2.2 Involvement of Serotonin in the plasticity of the CNS

Serotonergic neurons are found in a variety of organisms, from invertebrates to vertebrates, and in all species, the serotonergic network is highly plastic, showing changes in its anatomical organization throughout the life span of the organisms (Lillesaar and Gaspar 2019). It is noteworthy that, in addition to its well-established role as a neurotransmitter, 5-HT exerts morphogenic actions on the brain, influencing several neurodevelopmental processes such as neurogenesis, cell migration, axon guidance, dendritogenesis, synaptogenesis, and brain wiring (Wirth et al 2017).

During development, the brain of the fetus is influenced not only by endogenous 5-HT, but also by the contribution from the mother's placenta. Thus, the placenta represents a crucial micro-environment during neurodevelopment, contributing to the correct development of the CNS and

to long-term brain functions (Bonnin et al 2011). Therefore, maternal insults to placental microenvironment may alter embryonic brain development, resulting in prenatal priming of neurodevelopmental disorders (Shallie et al 2019). These pieces of evidence underline the critical importance of maintaining a correct 5-HT level during embryogenesis for proper brain circuit wiring and open a new perspective for understanding the early origins of neurodevelopmental disorders (Goeden et al 2016; Shah et al 2018; Brummelte et al 2017). Indeed, numerous studies on mice models showed that altered 5-HT level during development leads to neuro-pathologies. When the genes involved in 5-HT uptake or degradation are knocked out, the resulting increased 5-HT levels in the brain lead to altered topographical development of the somatosensory cortex and incorrect cortical interneuron migration (Cases et al 1996; Riccio et al 2009). On the other hand, the transient disruption of 5-HT signaling, during a restricted period of pre- or postnatal development, leads to long-term behavioral abnormalities in rodents, such as increased anxiety in adulthood (Ansorge et al 2008, Oberlander et al 2009). It is noteworthy that these animals do not show gross morphological alterations in the CNS suggesting that the lack of cerebral 5-HT may only affect the fine tuning of specific serotonergic circuits (Migliarini et al 2013). Interestingly, the transient silencing of SERT expression in neonatal thalamic neurons affects somatosensory barrel architecture through the selective alteration of dendritic structure and trajectory of late postnatal interneuron development in mouse cortex (De Gregorio et al 2019). Altogether, these findings indicate that perturbing 5-HT levels during critical periods of early development influences later neuronal development through alteration of CNS connectivity that may persist into the adulthood (Marín et al 2016; Teissier et al 2017). Interestingly, recent studies demonstrated that changes in 5-HT level affect axonal branch complexity, not only during development but also in adult life (Pratelli et al 2017).

Altogether, these data suggest that alterations in 5-HT signaling during brain development influence behavior and brain health also in adulthood. Accordingly, numerous studies have linked alterations of brain 5-HT signaling with psychiatric and neurodevelopmental disorders, such as major depression, anxiety, schizophrenia, obsessive compulsive disorder and Autism Spectrum Disorders (ASD; Shah et al 2018; Lesch and Waider 2012; Dayer 2014). In addition, since 5-HT plays a crucial role also in the maintenance of mature brain circuitry, it is intriguing to hypothesize that modulation of 5-HT signaling may rescue defects of CNS connectivity in the adult. For instance, the potential of 5-HT neurons to remodel their morphology during the entire life is indicated by the well-known capability of 5-HT axons of the adult to regenerate and sprout after lesions (Teissier et al 2017; Deneris and Gaspar 2018)

The effects of 5-HT during brain development, maintenance and dysfunction are mediated by at least 14 subtypes of receptors (5-HTRs), grouped in seven distinct families' classes (from 5-HT1R to 5-HT7R).

**2.3 Serotonin receptors**

All 5-HT receptors, grouped in seven distinct classes (from 5-HT1R to 5-HT7R), are broadly distributed in the brain. They display a highly dynamic developmental and region-selective expression pattern and trigger different signaling pathways. The current classification scheme considers not only operational criteria, such as drug-related characteristics, but also information about intracellular signal transduction mechanisms and amino acid sequence of the receptor protein (Hoyer et al, 1994; Hoyer and Martin 1997). All 5-HTRs, 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 (McCorvy et al 2015). Further receptor

heterogeneity is generated through alternative splicing (e.i. 5-HT3, 5-HT4 and 5-HT7 receptors), RNA editing (the 5-HT2C receptor), and the putative formation of homo- and heterodimers (Nichols and Nichols 2008; Prasad et al 2013). This great diversity of receptors indicates the wide and complex physiological roles of 5-HT in the nervous system (Pytliak et al 2011; Gellynck et al 2013).

**Table 1.** Classification of serotonin receptors

| Receptor Subtype | Distribution | Post-receptor mechanism |
|---|---|---|
| **5-HT1** (A-F) | Raphe nuclei, hippocampus, substantia nigra, globus pallidus, basal ganglia, cortex, putamen | $G_i/G_o$ –protein coupled, ↓ cAMP |
| **5-HT2** (A-C) | Cerebral cortex, hippocampus, substantia nigra, stomach fundus, choroid, platelets, smooth muscle, sensory and enteric nerves | $G_q/G_{11}$ –protein coupled, ↑ $IP_3$ |
| **5-HT3** | Area postrema, sensory and enteric nerves | Receptor is a $Na^+$ -$K^+$ ion channel |
| **5-HT4** | CNS and myenteric neurons, smooth muscle | $G_s$- protein coupled, ↑ cAMP |
| **5-HT5** (A, B) | Brain | $G_i/G_o$ –protein coupled, ↓ cAMP |
| **5-HT6** | Brain | $G_s$- protein coupled, ↑ cAMP |
| **5-HT7** | Brain | Gs- protein coupled, ↑ cAMP |

### 2.4 Serotonin receptor 7

The 5-HT7R, the last discovered member of the 5-HTRs family (Bard et al 1993, Ruat et al 1993), has been the subject of intense investigation, due to its high expression in functionally relevant regions of the brain (Matthys et al 2011). In mammalian CNS, the 5-HT7R is mainly expressed in the spinal cord, thalamus, hypothalamus, hippocampus, prefrontal cortex, striatal complex, amygdala and in Purkinje neurons of the cerebellum (Volpicelli et al 2014, Lippiello et al 2016, Crispino et al 2020). This wide distribution reflects the numerous functions in which the receptor is involved, such as circadian rhythms, sleep-wake cycle, thermoregulation, learning and memory processing, and nociception (Blattner et al 2019). In mammals, this receptor exhibits a number of functional splice variants due to the presence of introns in the 5-HT7R gene and to alternative splicing. The splice variants of the receptor, named 5-HT7(a), (b), (c) in rodents, and 5-HT7(a), (b), (d) in humans (Gellynck et al, 2013; Heidmann et al 1997,1998), display similar localization, ligand binding affinities, and activation of adenylate cyclase. To date, the only functional difference among the variants is that the human 5-HT7(d) isoform displays a different pattern of internalization compared to the other isoforms (Guthrie et al 2005). The 5-HT7R is a G protein-coupled receptor, that activates at least two different signaling pathways. The classical pathway relies on the activation of $G\alpha_s$ and the consequent stimulation of adenylate cyclase, leading to an increase in cyclic adenosine monophosphate (cAMP). The cAMP activates protein kinase A (PKA), that in turn phosphorylates various proteins such as the extracellular signal-regulated kinases (ERK) (Leopoldo et al 2011). Another 5-HT7R pathway depends on the activation of $G\alpha_{12}$, whose associated pathway leads to stimulation of Rho GTPases, Cdc42 and RhoA. In turn, these intracellular signalling proteins, that are critical for the regulation of cytoskeleton organization, lead to morphological modifications of fibroblasts and neurons (Kvachnina et al 2005). 5-HT7R

signaling also involves changes in intracellular $Ca^{2+}$ concentration and $Ca^{2+}$/calmodulin pathways (Lenglet et al 2002; Johnson-Farley et al 2005; Figure 1).

Recently, it has been shown that the 5-HT7R can form homodimers, as well as heterodimers with 5-HT1AR (Prasad et al 2019). Interestingly, 5-HT1AR signaling get suppressed when it forms heterodimers with 5-HT7R, while 5-HT7R signaling is not affected by heterodimerization with 5-HT1AR. Thus, oligomerization of these receptors may profoundly affect their downstream signaling, modulating several brain functions in which they are involved.

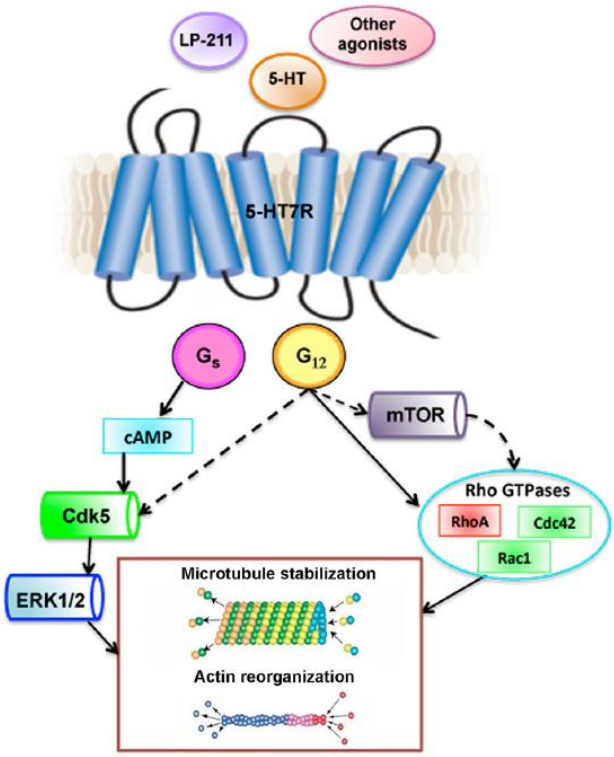



**Figure 1** Schematic drawing of 5-HT7R signaling pathways and downstream effectors leading to remodeling of neuronal morphology (from Volpicelli et al 2014)

### 2.5 Remodeling of Neuronal Circuits by 5-HT7R during Development

A wealth of data indicates that the activation of 5-HT7R modulates neuronal morphology and plasticity contributing to the establishment and maintenance of brain connectivity during embryonic and early postnatal life. To investigate the physio-pathological role of this receptor, the availability of selective agonists and antagonists was crucial, as well as of genetically modified mice lacking 5-HT7R (Hedlund et al 2003; Di Pilato et al 2014). Indeed, by using rodents' neuronal primary cultures of several brain areas, such as hippocampus, striatum and cortex, and various 5-HT7R agonists in combination with selective antagonists, it was consistently shown that pharmacological stimulation of the endogenous 5-HT7R promotes a pronounced extension of neurite length (Tajiri et al 2012; Rojas et al 2014, Speranza et al 2013; Lacivita et al 2016). The 5-HT7R-dependent neurite elongation was shown to rely on *de novo* protein synthesis and multiple signaling systems, such as ERK, Cdk5, Rho GTPase, Cdc42 and mTOR. These pathways converge to promote the reorganization of the neuronal cytoskeleton, which underlies the morphogenic effect of 5-HT7R stimulation (Speranza et al 2013, 2015). In addition, it has been recently reported that, in cultured mice hippocampal neurons, 5-HT7R mRNA is downregulated by miR-29a, which overexpression impairs 5-HT7R-dependent neurite elongation (Volpicelli et al 2019). These data point to miR-29a as a physiological modulator of 5-HT7R expression in the CNS that plays a key role in the subtle regulation of neuronal cytoarchitecture.

Interestingly, 5-HT7R activation has been demonstrated to modulate glutamate neurotransmission. Indeed, in hippocampal neurons, 5-HT7R activation modulates NMDA receptor activity (Vasefi et al 2013a,2013b), and increases AMPA receptor-mediated neurotransmission (Andreetta et al 2016). Accordingly, 5-HT7R-KO mice display reduced Long-Term Potentiation (LTP) in hippocampus (Roberts et al 2004). In addition, chronic stimulation of the 5-HT7R/$G_{12}$ signaling

pathway promotes dendritic spine formation, enhances basal neuronal excitability, and modulates LTP in organotypic slices prepared from juvenile mice hippocampus (Roberts et al 2004).

In view of the role played by 5-HT7R signaling pathways in remodeling neuronal morphology during development, it was not surprising that prolonged stimulation of this receptor, leading to the downstream activation of Cdk5 and Cdc42, increased the density of filopodia-like dendritic spines and synaptogenesis in cultured striatal and cortical mouse neurons (Speranza et al 2017). The involvement of constitutive 5-HT7R activity in shaping developing synapses was confirmed by data showing that in neuronal cultures with pharmacological inactivation of 5-HT7R, as well as in neurons isolated from 5-HT7R-deficient mice, the number of dendritic spines decreased (Speranza et al 2017). The involvement of 5-HT7R in spinogenesis and synaptogenesis, together with the demonstration that its activation is able to stimulate protein synthesis-dependent neurite elongation, as well as axonal elongation (Speranza et al 2013, 2015), suggests the intriguing possibility that the activation of this receptor may be linked to the axonal and synaptic system of protein synthesis (Giuditta et al 2008, Crispino et al 2014, Perrone-Capano et al 2021).

### 2.6 Remodeling of Neuronal Circuits in Adults by 5-HT7R

Neuronal circuits are able to reorganize in response to experience not only during development, but also in adulthood, showing plasticity along the entire life (Hübener and Bonhoeffer 2014). Consistently, the effect of 5-HT7R on neuronal plasticity are not restricted to embryonic and early postnatal development but extend also to later developmental stages and adulthood. Hence, it was shown that selective pharmacological stimulation of 5-HT7R during adolescence determines its persistent upregulation in adult rat forebrain areas (Nativio et al 2015). Stimulation of 5-HT7Rs in adolescent rats leads to increased dendritic arborization in the nucleus accumbens, a limbic area

involved in reward, as well as increased functional connectivity in different forebrain networks likely to be involved in anxiety-related behaviour (Canese et al 2015). Interestingly, the acute in vivo administration of a selective 5-HTR7 agonist improved cognitive performance in in Fmr1 KO mice, a model of Fragile X Syndrome (Costa et al 2018), suggesting that long-term changes of synaptic plasticity, which is the substrate of memory formation, requires neural network rewiring mediated by 5-HT7R. Accordingly, 5-HT7R-KO mice exhibit reduced hippocampal LTP, and specific impairments in learning and memory (Beaudet et al 2017; Roberts et al 2004). Interestingly, the expression level of 5-HT7R in the hippocampal CA3 region decreases with age (Beaudet et al 2015), suggesting that the spatial memory deficits associated with aging could be attributed to decreased 5-HT7R activity in this brain region. On the other hand, different studies have produced contradictory results with regards to the involvement of 5-HT7R in memory and attention-related processes (Freret et al 2014; Zareifopoulos and Papatheodoropoulos 2016), probably due to experimental differences (animal strain, behavioural tests, compounds and doses, route of administration, etc.). In conclusion, although the role of this receptor in cognitive functions is still controversial, it is possible to conclude that it modulates various aspects of learning and memory processes.

Altogether, these findings strongly suggest that the 5-HT7R plays a role in modulating synaptic plasticity and neuronal connectivity in both developing and mature brain circuits, although the molecular and cellular mechanisms underlying this modulation are only partially understood.

### 2.7 Presynaptic protein synthesis

Synaptic plasticity requires rapid and subtle modulations of the proteome. An always-growing body of evidence indicates that in neurons, highly polarized cells with complex arborisation and

remote synaptic terminals, proteins may be synthesized not only in the neuronal cell body, but also in synaptic regions in response to local signals. The on-site and on-demand synthesis of proteins has a crucial role in remodeling synaptic regions and highly contributes to synaptic plasticity (Giuditta et al 2008, Holt et al 2019, Perrone-Capano et al 2021). To study the local protein synthesis in presynaptic terminals, a very useful preparation is the synaptosomal fraction (Hernández et al., 1976; Whittaker, 1993). Synaptosomes are produced by mild homogenization of brain tissues followed by subcellular fractionation; they contain all the components of synaptic regions *in vivo* without the corresponding cell bodies (Whittaker, 1993). Using synaptosomes, as well as several other methodological approaches, it was demonstrated that synaptic translation plays a key role in controlling axonal shape, branching, and synaptogenesis during brain development, contributes to neuroplasticity and neurotransmission in mature brain, and its deregulation is involved in various neuropathologies (Holt et al 2019, Perrone Capano et al 2021). Thus, the intracellular pathways activated by serotonergic receptors are good candidates to be involved in the regulation of the synaptic system of protein synthesis.

**2.8 Alteration of Serotonin signaling in Autistic Spectrum Disorder**

Numerous genetic and pharmacological studies have linked defects of brain 5-HT signaling with psychiatric and neurodevelopmental disorders, such as major depression, anxiety, schizophrenia, obsessive compulsive disorder and Autism Spectrum Disorders (ASD) (Lesch and Waider 2012; Dayer 2014; Muller et al 2016, Garbarino et al 2019).

ASD refers to a heterogeneous group of neurodevelopmental disorders characterized by impaired social interaction and communication, repetitive and stereotyped behaviours, often accompanied by cognitive defects (Sztainberg and Zoghbi 2016). It has been widely demonstrated that the brain

5-HT neurotransmission system is altered in ASD patients, and in various animal models of the disease (Muller et al 2016; Garbarino et al 2019). For instance, mice lacking brain 5-HT have several typical abnormal phenotypes such as growth retardation and high aggressive behaviour, but also display selective deficits like impairment in social interactions and repetitive behaviour, which are features of ASD (Pratelli et al 2019; Mosienko et al 2019). Interestingly, it has been demonstrated that the ASD phenotype can depend on variants in the gene encoding the 5-HT transporter (SERT), an integral plasma membrane protein, present on presynaptic terminals of serotonergic neurons in the brain (Blakely et al, 1991, Qian et al, 1995). In addition, reduction of SERT binding capacity in the frontal cortex has been found in autistic children (Makkonen et al 2008). Accordingly, various clinical studies have investigated the therapeutic effects of selective serotonin reuptake inhibitors (SSRIs), which bind to SERT and block 5-HT uptake, in ASD and are widely used for the treatment of psychiatric disorders (Vaswani et al, 2003, Kellner, 2010). However, so far there is no consensus on the efficacy of SSRIs in ASD, probably due to the different pharmacokinetic and pharmacodynamic profiles of SSRIs, the extreme heterogeneity of ASD aetiology and the diversity of serotonin signaling systems in the brain (Nakai et al 2018).

Interestingly, 5-HT7R signaling was shown to be linked not only to ASD, but also to numerous neuropsychiatric and neurodevelopmental diseases characterized by impaired neuronal connectivity, such as Rett syndrome (RTT) and Fragile X syndrome (FXS; Costa et al 2018, De Filippis et al 2014, 2015). Moreover, a broad range of antidepressant and antipsychotic drugs display high affinity interactions with 5-HT7Rs (Leopoldo et al 2011). Although various pharmacological studies are providing evidence that targeting 5-HTRs has the potential to treat the core symptoms of ASD and the associated intellectual disabilities (Chugani et al 2016; De Bruin et al 2014), none of the approved ASD drugs are highly selective for 5-HT7R, limiting its potential

as a target for pharmacological treatment of ASD in humans. Nevertheless, brain-permeant and selective agonists of 5-HT7R have been successfully employed to treat ASD dysfunctions in animal models of FXS and RTT. In fact, activation of 5-HT7R is able to rescue synaptic plasticity, learning and autistic-like behaviour in adult FXS mice (Costa et al 2018), and improves cognitive and motor coordination deficits, as well as spatial memory, synaptic plasticity and mitochondrial dysfunction in RTT mice (De Filippis 2014, 2015, Valenti et al 2017). The recent demonstration of the presence of 5-HT7R on the mitochondrial membrane in neuroblastoma cell lines indicates the mitochondrial implication in the pathway associated to the receptor activation (Tempio et al 2020), suggesting the involvement of this receptor in diseases associated with mitochondrial dysfunction, such as ASD. Moreover, recent data showed that 5-HT7R stimulation can rescue brain histone acetylation abnormalities in RTT female mice, linking the activation of this receptor to epigenetic outcomes (Napoletani et al 2021).

These results have important therapeutic implications, indicating that the modulation of 5-HT7R may reverse severe behavioural and molecular deficits in animal models even in the adult (Figure 2).

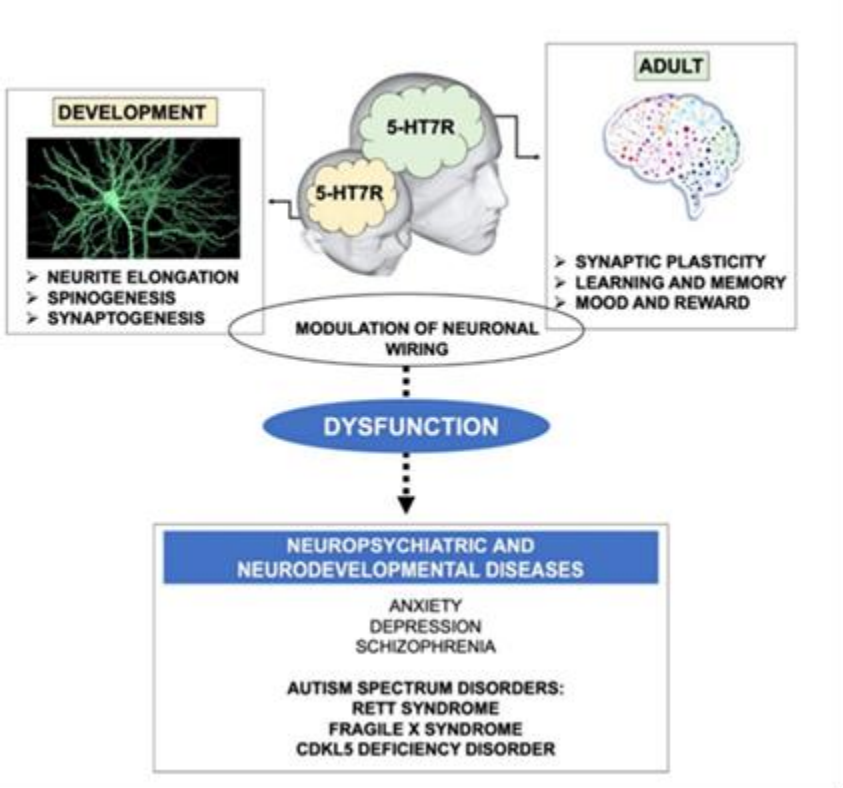



**Figure 2.** Schematic drawing illustrating the role of the 5-HT7R in brain plasticity during development and in the adulthood (from Crispino et al 2020)

### 2.9 5-HT7R ligands

In view of the implication of 5-HT7R signaling pathways in neurodevelopmental diseases characterized by altered connectivity, this receptor can be a potential candidate for a therapeutic target for the treatment of these pathologies.

Therefore, considerable efforts have been dedicated to the development of selective 5-HT7R agonists and antagonists (Leopoldo 2004; Pittalà et al., 2007). Several 5-HT7R antagonists are currently available, including selective antagonists such as SB-269970 (Hagan et al 2000). As for agonists, for a long-time unselective compound, such as 5-carboxamidotryptamine (5-CT), 5-

hydroxytryptamine (5-HT) or 8-hydroxy-2-(dipropylamino) tetralin (8-OH-DPAT) (a 5-HT1A/7R agonist) have long been used due to the absence of selective agonists of 5-HT7R (Nikiforuk et al 2015). The specificity of their effects was demonstrated using them in combination with selective antagonists. Therefore, numerous efforts were dedicated to developing a series of piperazine derivatives as potential 5-HT7R agonists (Badarau et al 2009; Intagliata et al 2016). Among these, N-(4-cyanophenylmethyl)-4-(2-diphenyl)-1-piperazinehexanamide (LP-211) displayed potent 5-HT7R agonist activity (Leopoldo et al., 2008) with pharmacodynamics and pharmacokinetic properties suitable for both *in vitro* and *in vivo* studies aimed to characterize the functional role of this receptor (Hedlund et al., 2010). As discussed above, a plethora of studies have shown that activation of 5-HT7R with LP-211 modulates neuronal connectivity (Speranza et al 2013, 2015, 2017, Volpicelli 19, Kvachnina et al 2005; Kobe et al 2012) and rescues behavioural and physiological alterations in animal models of many different neurological and neurodevelopmental diseases (Costa et al 2018; De Filippis 2015,2017; Napoletani et al 2021; Beaudet et al 2017; Carbone et al 2018; Santello et al 2017).

Thus, compounds such as LP-211 could represent an innovative pharmacological approach, for selectivity and efficacy, able to improve synaptic plasticity and the consequent compromised connectivity of neuronal circuits. To validate a pharmacological use of a new compound, however, it is necessary to study its molecular mechanisms of action and possibly test similar compounds to select those that have greater pharmacological efficacy and offer greater guarantees of use.

Over the years, the affinity and the potency of a drug candidate for a given target measured at equilibrium have been the sole parameters to guide the process of drug discovery. In recent years, the temporal aspects of drug-receptor interactions have been receiving growing interest. For instance, it was suggested that the association rate of a drug with its receptor ($k_{on}$) may be just as

important as the length of time the drug remains bound (residence time, $1/k_{off}$) in determining drug pharmacodynamics *in vivo* (Guo et al 2014; Copeland et al 2016). Indeed, the residence time of a drug is currently considered as one of the most important contributors to the biological efficacy of drugs *in vivo* (Guo et al 2014; Copeland et al 2016; Hoffmann et al 2015). The preference for drugs with "long" or "short" residence time may vary for different targets and for different therapeutic indications (Núñez et al 2012). Long residence time drugs offer advantages for those therapies requiring prolonged target occupancy so that the drug continues to exert its pharmacological effect even when most of the free drug has been already eliminated from the blood stream (Swinney et al 2009). On the other hand, long receptor occupancy can imply mechanism-based toxicity and, thus, a fast-dissociating drug is preferred.

### 2.10 Angelman Syndrome

Angelman syndrome (AS) is a rare incurable neurodevelopmental disorder characterized by severe intellectual disability, lack or impairment of speech, motor dysfunction, hyperactivity, autism-like behaviour, and seizure activities (Bi et al 2016, Margolis et al 15, Tan and Bird 2016). AS is reported to have a high comorbidity with ASD, especially with regards to the developmental delay and language impairment (Trillingsgaard and Østergaard, 2004; Hogart et al, 2010). Developmental delay in AS patients is usually noticed within the first year of life, with language skills more delayed than motor skills, excessive inappropriate laugher and sleep disturbance (Bird 2014).

AS is characterized by the lack of expression in the brain of the maternally inherited Ube3a gene located on chromosome 15q11.2 (Tan and Bird 2016). Neurons entirely depend on Ube3a expressed from the maternal allele, since the paternally inherited copy of the gene is silenced, in a

process named genetic imprinting. The paternal Ube3a expression is repressed by an atypical RNA polymerase II transcript (Bi et al 16, Meng et al 12). Because of this epigenetic imprinting, the deletion or mutation of the Ube3a gene in maternal chromosome leads to the total loss of the transcript.

The Ube3a gene encodes ubiquitin-protein ligase E3A, which belongs to the family of E3 enzymes, which regulate the process of protein ubiquitylation, one of the key posttranslational protein modifications (Rotin and Kumar, 2009). Ubiquitylation, or tagging proteins with ubiquitin, targets protein for degradation by the proteasome complex, but also controls intracellular signaling and protein subcellular localization. Ubiquitylation is a sequential process: in the first step the ubiquitin is activated, in an ATP-dependent way, by the activating-enzyme E1. The complex ubiquitin-E1 is transferred to ubiquitin-conjugating enzyme (E2), and then to a ubiquitin–protein ligase (E3), which facilitates the transfer of ubiquitin to lysine residues in the substrate. Interestingly, it has been demonstrated that UBE3A functions not only as an E3 ligase in the ubiquitin-proteasome pathway (Greer et al 10), but also as a transcriptional co-activator for steroid hormone receptors (Nawaz et al 99, Ramamoorthy and Nawaz 08). However, it is not clear if both of these functions, when altered, are responsible for the phenotype of AS.

While deficiency in the maternal copy of Ube3a is causing AS (Buiting et al 16, Bird 2014, Williams et al 10), duplication or increased expression of the gene is linked to ASD (Smith et al, 2011; Urraca et al, 2013). Indeed, Ube3a has been demonstrated to play a key role in neural circuit maturation and experience-dependent plasticity in the mammalian cerebral cortex (Yashiro et al, 2009; Sato and Stryker 2010).

An animal model of AS widely used is a mouse strain with maternal Ube3a deficiency, which exhibits AS features, such as motor dysfunction, inducible seizures, and learning and memory

deficits, similar to the symptoms of AS individuals (Jiang et al 98, van Woerden et al 07). AS mice also display impairment in LTP, a postulated molecular and cellular mechanism underlying learning and memory (Baudry et al 12, Kaphzan et al 12, Bi et al 2016).

### *2.10.1 Serotonin signaling in Angelman Syndrome*

Despite this comorbidity and the demonstrated involvement of 5-HT in ASD, only few studies have focused directly on the link between 5-HT and AS. One of these studies demonstrated that 5-HT levels were elevated in the striatum and cortex of maternal deficient Ube3a mouse compared to controls (Farook et al 2012). On the other hand, no significant changes of 5-HT levels were observed in the brain of maternal Ube3a duplication mouse, which is a model for ASD. These results seem to indicate that the modulation of 5-HT in the brain does not depend exclusively on Ube3a expression levels (Farook et al 2012).

In a different study it was demonstrated that chronic treatment of AS mice with the SSRI fluoxetine attenuates the molecular and behavioural deficits of the animals (Godavarthi et al 2014). These data emphasize the key role played by the serotonergic system in the pathogenesis of AS. Accordingly, a recent study indicated that buspirone, a partial agonist of 5-HT1AR, was able to ameliorate anxiety-related behaviours of AS human patients (Balaj et al 2019). Given the role of the 5-HT7R in plasticity mechanisms and its involvement in ASD, it would be not surprising if many of the numerous altered molecular mechanisms linked to AS may be modulated by this receptor.

### *2.10.2 Molecular mechanisms underlying altered synaptic plasticity in AS: involvement of 5-HT7R*

The molecular mechanisms underlying AS has been object of intense investigation, with special attention dedicated to the processes involved in the altered synaptic plasticity associated with AS (Bi et al 2016). In addition to impaired LTP and smaller brains, AS mice exhibit reduced number and size of dendritic spines in neurons of diverse brain regions including cerebellum, hippocampus and visual cortex (Dindot et al 2008, Sun et al 2015a, Yashiro et al 2009). Accordingly, a post-mortem study in AS patients revealed a reduced spine density of pyramidal neurons in the visual cortex (Jay et al 1991). To understand whether the spine abnormalities are derived from decreased spine formation and/or increased spine elimination, an elegant study was performed using *in vivo* time lapse two-photon microscopy to evaluate, over multiple days, the number, dynamic, and morphology of pyramidal neuron dendritic spines in the primary visual cortex of control and AS model mice (Kim et al 2016). It was found that, in neurons of AS mice exposed to visual experience, spine formation was comparable to wild type animals, but their elimination was greatly enhanced. However, when AS animals were exposed to light deprivation, spine density and turnover were indistinguishable from those of wild-type mice. These data indicated that the decreased spine density in AS model mice reflects impaired experience-dependent spine maintenance (Kim et al 2016). Interestingly, it was recently demonstrated that in AS hippocampal neurons, loss of UBE3A was linked to axonal guidance deficit and increased axonal branching, depending on altered cytoskeleton dynamics. Altogether, these data emphasized the role of defective neuronal connectivity and plasticity in the pathogenesis of AS (Tonazzini et al 2019). One of the proteins involved in AS altered synaptic plasticity is Arc (activity-regulated cytoskeletal protein), one of the first identified Ube3a target proteins in mammalian brain (Greer et al 2010).

Indeed, Arc is known to be ubiquitinated and degraded in an Ube3a-dependent manner, although it was also indicated that Ube3a modulates Arc at the transcriptional level (Kuhnle et al 13). Arc is able to stimulate the internalization of the AMPA receptors (AMPAR), leading to reduction of AMPAR-mediated synaptic transmission. Thus, the impairment of LTP in AS may depend on increased Arc levels due the lack of Ube3a (Rial Verde et al 2006). Interestingly, it was demonstrated that activation of 5-HT7R increases the phosphorylation of the GluA1 subunit of AMPA receptors, facilitating AMPA receptor-mediated neurotransmission (Andreetta et al 2016). These data indicate that 5-HT7R is a possible candidate to mediate the rescuing of impaired LTP in AS.

It is noteworthy that LTP induction and maintenance depends on Brain-Derived Neurotrophic Factor (BDNF)-induced signaling through its receptor, TrkB, which is known to interact with the postsynaptic density protein-95 (PSD-95) (Cao et al 2013). Since Arc is also interacting with PSD-95, in AS mice, which are characterized by increased levels of Arc, PSD-95 is abnormally sequestered by Arc. As a consequence, the recruitment of PSD-95 by TrkB is impaired in these mice, and BDNF signalling is altered (Cao et al 2013). 5-HT7R activation has been demonstrated to promote an increase in TrkB receptor expression and phosphorylation (Samarajeewa et al 2014). Thus, this altered molecular pathway in AS might also be rescued by modulation of 5-HT7R signaling.

LTP at hippocampal CA3/CA1 synapses is influenced by small conductance calcium-activated potassium channels (SK2), which are also involved in learning, memory, and synaptic plasticity (Hammond et al 2006, Lin et al 2008). SK2 channels are localized in dendritic spines where their activity regulates calcium influx through NMDA receptors (NMDAR). Indeed, during an excitatory postsynaptic potential (EPSP), calcium influx opens SK2 channels in individual spines.

The repolarizing potassium current reduces the EPSP and triggers the blockage of NMDAR by magnesium. As a consequence, blocking SK2 channels facilitates the induction of LTP (Ngo-Anh et al 2005). Interestingly, it was demonstrated that SK2 protein is ubiquitinylated by Ube3A in its C-terminal domain, and that ubiquitination facilitates its internalization (Sun et al 2015a). In AS mice, the lack of Ube3A results in increased SK2 levels, leading to rapid repolarization of the membrane following synaptic transmission, thus limiting the activation of NMDARs, and ultimately impairing LTP. Accordingly, the selective blockage of SK2 channels with apamin was able to reverse the impairment of LTP and learning performance of AS mice in a fear conditioning paradigm (Sun et al 2015a). In addition, apamin was also able to block the enhanced low-frequency-induced LTD observed in hippocampal slices from AS mice. These apamin rescuing effects on LTP impairment and LTD enhancement were both NMDAR-dependent (Sun et al 2015a). It is noteworthy that activation of 5-HT7R is able to modulate glutamate neurotransmission in hippocampus affecting both NMDAR and AMPAR (Costa et al. 2012,Vasefi et al 2013a, Andreetta et al 2016), making 5-HT7R a possible target for rescuing AS impairment of synaptic plasticity.

The cerebellum of AS mice presents abnormalities in dendritic spine morphology, number and length (Dindot et al 2008), and were linked to altered signaling of the mechanistic target of rapamycin (mTOR) pathway (Sun et al 2015b). The mTOR pathway regulates major cellular functions, controlling cell growth and proliferation (Laplante and Sabatini 2012), and plays critical roles in neuronal cells during development and in synaptic plasticity, since it is involved in several processes, such as transcription, ubiquitin-dependent proteolysis, and microtubule and actin dynamics (Takei et al 2004, Jaworski and Sheng, 2006, Hoeffer and Klann 2010). mTOR, coupled with different accessory proteins, forms two distinct multiprotein complexes, mTORC1 and

mTORC2, differently sensitive to rapamycin (Takei and Nawa, 2014). In the cerebellum of AS mice, the imbalanced signaling of the mTOR pathway results in increased mTORC1 and decreased mTORC2 activation (Sun et al, 2015b). Interestingly, treatment with rapamycin normalized both mTORC1 and mTORC2 signaling and improved dendritic spine morphology of Purkinje neurons and motor function in AS mice. A similar abnormal mTOR signaling, reversed by rapamycin treatment, was demonstrated to be responsible for altered hippocampal synaptic plasticity and fear-conditioning memory in AS animal model (Sun et al 2016). These data underlined the crucial involvement of mTOR signaling dysregulation in AS pathogenesis (Sun et al 2015b). It is worth noticing that stimulation of 5-HT7R with LP-211 in neuronal primary cultures from different regions of embryonic mouse brain selectively activate mTORC1 signaling and other signal transduction pathways eventually converging on cytoskeleton reorganization and consequent axonal elongation (Speranza et al 2015). In addition, LP-211 treatment of postnatal cortical and striatal neurons from mouse brain promotes the formation of dendritic spines and facilitates synaptogenesis (Speranza et al 2017). These data suggest that modulation of 5-HT7R may be considered as a potential treatment to reverse the morphogenic impairments observed in AS (Table 2).

**Table 2** Pathways altered in AS and modulated by 5-HT7R

| **Angelman syndrome** | **5-HT7R** |
|---|---|
| Reduction of synaptic transmission mediated by AMPA receptors (Kuhnle et al 2013) | Facilitation of AMPA receptor mediated neurotransmission (Costa et al. 2012, Andreetta et al 2016) |
| Limited activity of NMDA receptors (Sun et al 2015a) | NMDA receptor modulation (Costa et al. 2012,Vasefi et al 2013) |
| Alteration of TrkB and the BDNF pathway (Cao et al 2013) | Increased expression and phosphorylation of TrkB (Samarajeewa et al 2014) |
| Imbalanced signaling of the mTOR pathways (Sun et al, 2015b) | Modulation of mTOR pathways (Speranza et al 2015) |

## 3. AIMS

Serotonin is a neurotransmitter that modulates numerous physiological aspects of the nervous system. Among the different subtypes of serotonin receptors, 5-HT7R plays a key role in neuronal plasticity by modulating neuronal cytoarchitecture and connectivity during development and in adulthood, through various signaling mechanisms and molecular pathways (Leo et al, 2009, Volpicelli et al, 2014). As a consequence, dysfunction of these signaling pathways could be implicated in neurodevelopmental diseases characterized by impaired neuronal connectivity and associated with cognitive dysfunctions. From this point of view, 5-HT7R can be considered as a good candidate for a therapeutic target for the treatment of these pathologies, and its selective ligands could represent a possible pharmacological approach to ameliorate altered synaptic plasticity and connectivity. However, to validate the pharmacological use of a new compound, it is necessary to test similar compounds for safety and pharmacological efficacy.

The first aim of my work has been the characterization of additional selective ligands for 5-HT7R by measuring their residence time (RT), a pharmacodynamic parameter that can be combined with commonly used parameters such as affinity, maximum response, and potency. In particular, the residence time seems to be more correlated with the *in vivo* efficacy of a drug than the receptor affinity measured under equilibrium conditions. Previous studies suggested that the residence time of a set of agonists of cannabinoid CB2 receptor was structure-dependent. Thus, it was evaluated if the residence time of the 5-HT7R ligands was related to the lipophilicity of these compounds.

The different residence time of 5-HT7R ligands could translate into different pharmacological properties of the compounds themselves. Thus, the second aim of my work was to verify the effects of 5-HT7R ligands with different residence times on neurite elongation of primary neurons in

culture. The effects of these novel ligands were compared to the 5-HT7R agonist LP-211, which is able to promote neurite outgrowth (Speranza et al 2013, 2015).

The third aim of my work was to investigate the effect of 5-HT7R activation on synaptic plasticity, using brain synaptosomes from adult rodents as *in vitro* model of a locally regulated synapse. We stimulated synaptosomes from various brain areas involved in higher cognitive functions with LP-211 to study the effects on some of the plastic events occurring at the synapse, such as secretion mechanism and local protein synthesis.

As a fourth aim, we evaluated the ability of LP-211 to revert some of the altered phenotypes in the AS mice model. Since many molecular mechanisms altered in AS were positively regulated by 5-HT7R, it is possible to conclude that 5-HT7R can be a novel therapeutic target for the treatment of AS.

# 4. MATERIALS AND METHODS

## 4.1 Characterization of the pharmacokinetic properties of 5-HT7R ligands

### *4.1.1 Drugs and reagents*

Cell culture reagents were purchased from Euro Clone (Milan, Italy). G418 (geneticin) and 5-HT were purchased from Sigma-Aldrich (Milano, Italy). 5-CT and SB-269970 were purchased from Tocris Bioscience (Bristol, UK). [$^3$H]-5CT was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). MultiScreen plates with Glass fiber filters were purchased from Merck Millipore (Billerica, MA, USA).

A set of 5-HT7R ligands previously reported by Marcello Leopoldo (Biofordrug srl, Italy) and co-workers (Hansen et al 2014, Lacivita 2020) were selected for further analysis:

- LP-211 (N-(4-Cyanophenylmethyl)-4-(2-diphenyl)-1-piperazinehexanamide tartrate)
- 3-[4-(2-Biphenyl)piperazin-1-yl]-1-phenoxy-2-propanol (**1**)
- 1-[4-[3-(4-Fluorophenyl)pyridin-2-yl]piperazin-1-yl]-3-phenoxypropan-2-ol (**2**)
- 1-[4-(2-Biphenyl)piperazin-1-yl]-3-(4-methoxyphenoxy)propan-2-ol (**3**)
- 1-(4-Methoxyphenoxy)-3-[4-[3-(3-pyridyl)-2-pyridyl] piperazin-1-yl]-2-propanol (**4**)
- 1-(4-Methoxyphenoxy)-3-[4-[3-(4-pyridyl)-2-pyridyl] piperazin-1-yl]-2-propanol (**5**)
- 1-(4-Methoxyphenoxy)-3-[4-[2-(3-pyridyl)-3-pyridyl] piperazin-1-yl]-2-propanol (**6**)
- 1-(4-Methoxyphenoxy)-3-[4-[2-(4-pyridyl)-3-pyridyl] piperazin-1-yl]-2-propanol (**7**)
- 1-[4-[2-(4-Methoxyphenyl)phenyl]piperazin-1-yl]-3-(2- pyrazinyloxy)-2-propanol (**8**)
- 2-[4-[4-[2-(4-Methoxyphenyl)phenyl]piperazin-1-yl]butoxy]benzonitrile (**9**)
- (*R*)-1-[4-(Imidazo[1,2-a]pyridin-2-yl)phenoxy]-3-[4-[2-(4-methoxyphenyl)phenyl]piperazin-1-yl]propan-2-ol (**10**)

- 6-[(*R*)-2-Hydroxy3-[4-[2-(4-methoxyphenyl)phenyl]piperazin-1-yl]propoxy]-2-methyl-2H-benzo[b][1,4]oxazin-3(4H)-one (**11**)
- (*R*)-1-[4-[2-(4-Methoxyphenyl)phenyl]piperidin-1-yl]-3-(pyrazin-2-yloxy)-2-propanol (**12**)

### *4.1.2 Cell culture*

The HEK-293-5-HT7AR transfected cell line, which overexpresses serotonin receptors 7A was developed in Marcello Leopoldo's lab (Lacivita et al 2014). Transfected cells were grown in DMEM high glucose supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 µg/mL G418, in a humidified incubator at 37 °C with a 5 % CO2 atmosphere.

### *4.1.3 Competition Binding Assay*

5-HT7R competition binding assay was carried out as previously reported (Lacivita et al 2014). The experiments were performed in MultiScreen plates (Merck Millipore) with Glass fiber filters (GF/C), presoaked in 0.3% polyethylenimine for 20 min. After this pre-treatment, 130 µg of HEK-293-5-HT7AR membranes, 1 nM [$^3$H]-5-CT, and reference or test compounds were suspended in 0.25 mL of incubation buffer (50 mM Tris-HCl pH 7.4, 4 mM MgCl2, 0.1%, ascorbic acid, 10 µM pargyline hydrochloride), and incubated for 60 min at 37 °C. The incubation was stopped by rapid filtration and the filters were washed with 3 × 0.25 mL of ice-cold buffer (50 mM Tris-HCl, pH 7.4). Nonspecific binding was determined in the presence of 10 µM 5-CT. Approximately 90% of specific binding was determined under these conditions. Concentrations required to inhibit 50% of radioligand specific binding ($IC_{50}$) were determined by using six to nine different concentrations

of the compound studied in two or three experiments with samples in duplicate. Apparent inhibition constants (Ki) values were determined by nonlinear curve fitting, using the Prism, version 5.0, GraphPad software.

### *4.1.4 Association binding assay*

To determine constant affinity ($k_{on}$) of [$^3$H]-5CT, the observed association rate constant ($k_{ob}$) was measured by association assay. The experiment was performed in MultiScreen plates (Merck Millipore) with Glass fiber filters (GF/C), presoaked in 0.3% polyethylenimine for 20 min. After this pre-treatment, 130 µg of HEK-293-5-HT7AR membranes and 1 nM [$^3$H]-5CT were suspended in 0.25 mL of incubation buffer (see above) and incubated at 37 °C for different times (0, 5, 10, 15, 20, 30, 60, 90 min). The incubation was stopped by rapid filtration and the filters were washed with 3 × 0.25 mL of ice-cold buffer (Tris-HCl 50 mM, pH 7.4). Data were fitted using a one phase exponential association function by Prism, version 5.0, GraphPad software, to get the $k_{ob}$. The obtained $k_{ob}$ was used to calculate $k_{on}$ value by the following equation:

$$k_{on} = (k_{ob} - k_{off})/ [L]$$

in which [L] is the exact concentration of [$^3$H]-5-CT determined per each experiment.

### *4.1.5 Dissociation binding assay*

Dissociation rate ($k_{off}$) of [$^3$H]-5-CT was assessed by dissociation assay. The experiment was performed in MultiScreen plates (Merck Millipore) with Glass fibre filters (GF/C), presoaked in 0.3% polyethylenimine for 20 min. After this pre-treatment, 130 µg of HEK-293-5-HT7AR membranes and 1 nM [$^3$H]-5-CT were suspended in 0.25 mL of incubation buffer (see above) and

incubated at 37 °C for 30 min. After this equilibrium time, 10 µM 5-CT was added and the dissociation rate was measured at different times (0, 5, 10, 15, 20, 30, 60, 90 min). The incubation was stopped by rapid filtration and the filters were washed with 3 × 0.25 mL of ice-cold buffer (50 mM Tris-HCl, pH 7.4). Dissociation rate ($k_{off}$) value was determined by nonlinear curve fitting, using the Prism, version 5.0, GraphPad software.

#### *4.1.6 Competition association assay*

To determine the $k_{on}$ and $k_{off}$ values of selected ligands, all compounds were tested at their respective Ki. The experiments were performed in MultiScreen plates (Merck Millipore) with Glass fiber filters (GF/C), presoaked in 0.3% polyethylenimine for 20 min. After this pre-incubation, 130 µg of HEK-293-5-HT7AR membranes, 1 nM [$^3$H]-5-CT and reference or test compounds at their Ki were suspended in 0.25 mL of incubation buffer (see above). The samples were incubated at 37 °C for different times (0, 5, 10, 15, 20, 30, 60, 90 min). The incubation was stopped by rapid filtration and the filters were washed with 3 × 0.25 mL of ice-cold buffer (50 mM Tris-HCl, pH 7.4). $k_{on}$ and $k_{off}$ values were determined by nonlinear curve fitting, using the Prism, version 5.0, GraphPad software.

### 4.2 Neuronal primary cultures

#### *4.2.1 5-HT7R ligands*

The selective 5-HT7R agonist LP-211 and the 5-HT7R ligands, compound **4** and **6**, were provided by Marcello Leopoldo (see above).by  All compounds were used at a concentration of 100 nM, at days *in vitro* (DIV) indicated in the results section or in the figure legend and incubated for the appropriate time.

### *4.2.2 Neuronal primary cultures from mouse embryos*

Pregnant C57 BL/6 mice were housed, cared for and sacrificed in accordance with the European Commission recommendations (EU Directive 2010/63/EU for animal experiments). The animals were bred in-house at the Institute of Genetics and Biophysics 'Adriano Buzzati Traverso', CNR, Naples, Italy. All the procedures related to animal treatments were approved by the Ethic-Scientific Committee for Animal Experiments. The appearance of vaginal plug was considered as day E0 and the embryos were used at the embryonic age 18 (E18). Primary cultures were prepared from hippocampi of mouse embryos, as previously described (di Porzio et al.,1980, Speranza et al 2013, 2015). About 15–20 embryos from different dams were pooled for each cell culture preparation. The hippocampi 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 were dissociated from the tissue and 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 20 U/ml papain (Warthington, Milan, Italy) solution in Earle's balance salts containing 1 mM EDTA (Sigma-Aldrich, Milan, Italy), 1 mM cysteine (Sigma-Aldrich, Milan, Italy) and 0.01% pancreatic DNAse (Sigma Aldrich, Milan, Italy). After addition of 1 mg/ml of bovine serum albumin (Sigma-Aldrich, Milan, Italy) and 1 mg/ml ovomucoid (Sigma-Aldrich, Milan, Italy) cell suspensions were centrifuged at 800 g for 5 min, resuspended in plating medium and cell density evaluated (Fiszman et al., 1991). For viable cell count, cell suspension was diluted 1:10 with 0.1% trypan blue dye (Sigma-Aldrich, Milan, Italy) 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 $cm^2$ LabTek chamber slides (Nunc, Milan, Italy) for morphological analyses, and at density of $3 \times 10^5/cm^2$ in

9,5 $cm^2$ cell culture dishes (Corning, New York, USA) for RNA purification and Western blot analyses. Both chamber slides and cell cultured dishes were coated with15 µg/ml of poly-D-Lysine dissolved in water (Sigma-Aldrich, Milan, Italy). Cultures were grown in serum free Neurobasal medium (Life technologies, Milan, Italy), supplemented with B27 (Life technologies), 2 mM L-glutamine (Sigma-Aldrich, Milan, Italy), 50 U/mL penicillin (Sigma-Aldrich, Milan, Italy) and 50 µg/ml streptomycin (Sigma- Aldrich, Milan, Italy). Cells were maintained for 3 DIV at 37 °C in a humidified incubator in presence of 5% $CO_2$, before experimental manipulation. Each experimental point was performed from 3 independent cell preparations, and each neuronal culture was technically replicated three times.

### *4.2.3 Neuronal primary cultures from mouse pups*

C57 BL/6 mice were housed and sacrificed in accordance with the recommendations of the European Commission (EU Directive 2010/63/EU for animal experiments). The animals were bred in-house at the Institute of Genetics and Biophysics 'Adriano Buzzati Traverso', CNR, Naples, IT. All the procedures related to animal treatments were approved by the Ethic-Scientific Committee for Animal Experiments. Primary cultures were prepared from different brain regions of mouse pups at postnatal day 1 (P1) or postnatal day 3 (P3), as previously described (Speranza et al 2017, Volpicelli et al., 2019). Pups of both sexes were used. Brains were quickly isolated from mouse pups under sterile conditions and placed in HBSS (Cat. No. 24020-091; ThermoFisher Scientific, Milan, Italy). The areas of interest, striatum (STR), cortex (CTX) and hippocampus (HPP), were dissected from the brain under a stereomicroscope in HBSS with 10% fetal bovine serum (FBS, Euroclone, Milan, Italy) and then placed in HBSS without serum. The collected tissues were enzymatically dissociated by incubation for 90 sec at 37 °C in 0.1% trypsin (Sigma, Milan Italy)

in HBSS solution containing 0.01% pancreatic DNAse (Sigma, Milan Italy). Enzymatic dissociation was blocked by replacing the medium with HBSS containing $Ca2^{+}/Mg2^{+}$ and 10% fetal bovine serum (FBS) medium. Cells were washed in HBSS $Ca^{2+}/Mg^{2+}$ and mechanically dissociated by pipetting 10 times in 1 ml of Neurobasal A medium (NBM-A) containing 0.01% DNase. After a briefly centrifugation for 5 min at 500 rpm, cells were resuspended in 1 ml of NBM-A and their concentration was determined on the basis of the total cell count after trypan blue dye exclusion. Dissociated cells were plated in NBM-A medium supplemented with B27 (ThermoFisher Scientific) and 5% FBS, 2 mM Glutamax (ThermoFisher Scientific), 50 U/mL penicillin and 50 mg/mL streptomycin (ThermoFisher Scientific) at a density of 35 x $10^3/cm^2$ onto sterilized 12 mm coverslips (Corning Optical Communications S.r.l., Torino, Italy) freshly coated with 15 µg/mL of poly-D-lysine (Sigma-Aldrich, Milan, Italy). After 1 DIV, FBS was withdrawn and every third DIV half of the medium was replaced by fresh medium without FBS. Cultures were maintained at 37 °C and 5% $CO_2$ in a humidified incubator for 3-4 days. Each experimental point was performed from 3 independent cell preparations, and each neuronal culture was technically replicated three times.

### *4.2.4 Immunofluorescence and morphological analyses*

For morphological analyses, embryonic and postnatal cultures were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature, washed three times in PBS and permeabilized for 15 min in PBS containing 0.1% Triton-X-100. Neurons were treated for 30 min at room temperature in blocking solution [3% bovine serum albumin (BSA) in PBS] and then incubated overnight at 4 °C with a monoclonal antibody against neuron specific class III ß-tubulin (Tuj1; Sigma-Aldrich T8660, 1:750). Then, cells were washed in PBS and incubated for 2 h at

room temperature with fluorescent-labelled secondary antibodies (Alexa Fluor 594, 1:400; ThermoFisher Scientific) in PBS with 1% BSA. After washing, cells were stained with 4',6-diamidino-2-phenylindole (DAPI; nuclear stain, 1:1000) for 10 min at 22 °C and mounted on coverslips with oil mounting solution (Mowiol, Sigma-Aldrich). To evaluate neurite length, fluorescent signals from Tuj1 stained neurons were detected with a microscope (Leica DM6000B, Wetzlar, Germany) equipped with a 20x objective. Images were acquired with high-resolution camera using the software Leica Application Suite and analysed by the image-processing software ImageJ (https://imagej.net/Welcome). The length of neurites was measured manually using the software ImageJ starting from the perimeter of the soma. Only clearly visible cells were subjected to analysis to prevent inaccurate scoring (Speranza et al. 2015). A total of 10–15 fields for each cell-culture condition was used from at least three independent culture wells. The analyses were carried out "blind" to avoid any subjective influences during measurements.

### 4.3 Synaptosomes from rat brain

#### *4.3.1 Animals*

Male Wistar rats and (Charles River, Calco, Lecco, Italy) of about 3 months of age were kept in animal house at the Department of Biology (University of Naples Federico II), in a temperature-controlled room under a 12-h light-dark regimen (lights on at 6 AM) with food and water ad libitum. All animal procedures reported herein were approved by the Institutional Animal Care and Use Committee (CSV) of University of Naples Federico II. The animals were anesthetized by intra-peritoneal injection of chloral hydrate (40 mg/100 g body weight) and decapitated with a guillotine. The brain was removed, and cortex and cerebellum were quickly dissected and used immediately for synaptosomal preparation.

### *4.3.2 Preparation of Synaptosomal Fractions*

Synaptosomal fractions were prepared as previously described (Eyman et al., 2007). Briefly, cerebral cortex (Cx), and cerebellum (Cb) were rapidly dissected from rat brain and homogenized in 9 volumes of cold isotonic medium (HM: 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4), using a Dounce homogenizer. After centrifugation of the homogenate (2,000 *g*, 1 min, 4 °C), the sediment was resuspended in the same volume of HM and centrifuged under the same conditions to yield a washed sediment containing nuclei, cell debris, and other particulates (P1 fraction). The two supernatant fractions were mixed and centrifuged at a higher speed (23,000 *g*, 4 min, 4 °C), to obtain a second sediment that was resuspended in the same volume of HM and centrifuged as described above. The washed sediment contained free mitochondria, synaptosomes, myelin, and microsomal fragments (P2 fraction). An aliquot of the P2 fraction (1 ml), with protein concentration of 3..5 mg/ml was layered over a discontinuous gradient of 5% and 13% Ficoll dissolved in HM (2 ml each) and centrifuged at 45,000 *g*, 45 min, 4 °C. The purified synaptosomal fraction was recovered at the interface between the two Ficoll layers, diluted with 9 volumes HM and sedimented by centrifugation at 23,000 *g*, 20 min, 4 °C. The sediment was homogenized in HM and used for subsequent analyses.

### *4.3.3 Incubation of synaptosomal fractions*

To analyse the effect of LP-211 on synaptosomal protein synthesis and secretion, synaptosomes were incubated in Ringer medium (90 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM glucose, 100 mM sucrose, 30 mM mM Tris-Cl, pH 7,5) in presence of 100 nM LP-211 (in DMSO). As a negative control, a synaptosomal sample was incubated in a Ringer medium containing DMSO.

The reaction was started by adding the synaptosomal fraction (300 µg/ml) to the incubation medium. After 2 h incubation at 37 °C, the reaction was stopped by cooling the sample in ice. Synaptosomes were collected by centrifugation at 23,000 *g*, 10 min, 4 °C. The pellet, containing synaptosomal proteins, was resuspended in RIPA Buffer (50 mM Tris-HCl pH 7.8, 150 mM NaCl, 0.1% SDS, 0.5% NP-40, 0.5% DOC; protease and phosphatase inhibitor cocktail, Sigma-Aldrich, Milan, Italy), clarified by centrifugation in an Eppendorf 5415C microcentrifuge at 14,000 rpm, 5 min, 4 °C, and stored at -80 °C for subsequent western blot analysis.

The supernatant, containing the secreted proteins, was concentrated by Amicon Centrifugal Filter Devices with a cut-off of 10 kDa (Merck-Millipore), and stored at -80 °C for subsequent western blot analysis.

The protein concentration of the samples was determined by the Bradford assay (BIORAD).

### *4.3.4 Gel electrophoresis and Western blot analysis*

Aliquots of homogenates were lysed in RIPA buffer and clarified by centrifugation in Eppendorf 5415C microcentrifuge at 14,000 rpm, 5 min, 4 °C. Proteins from homogenates and synaptosomes and secreted proteins were separated on 10–15% SDS-polyacrylamide gel (5 µg/lane, in Sample Buffer: 60 mM Tris-HCl pH 6.8, 10% Glycerol, 2% SDS, 100 mM DTT 0.1% bromophenol blue) and transferred to PVDF membranes (Merck-Millipore). For western blot analysis, filters were blocked for 2 hrs at room temperature in 5% (w/v) non-fat milk in Tris-buffered saline Tween-20 (TBST; 0.1% Tween, 150 mM NaCl, 10mM Tris-HCl, pH 7.5) and probed for 1 h at 4 °C with the following antibodies: ab anti-synaptotagmin (1:1.000, Elabscience); ab anti- 5-HT7R (1:1,000, Novusbio), ab anti-Tau (1:20.000, Elabscience); ab-anti-CD81 (1:200, Santa Cruz); ab anti-α-tubulin (1:600,000, Abcam); ab anti-β-actin (1:2,000, BD Biosciences); ab anti-cystatin-B

(1:4,000, Antibodies); ab anti-eIF4G2 (1:1,000, SIGMA); ab anti-eIF2B2 (1:1,000, SIGMA), ab anti-synaptophysin (1: 400,000, Merck-Millipore). Overnight incubations were performed for ab anti-ERK and anti-p-ERK (1: 1,000, Cell Signaling), ab anti Akt and anti p-Akt (1:1,000, Cell Signaling), ab anti P70 and p-P70 (1:1.000, Cell Signaling). After several washes in TBST, filters were incubated with a secondary antibody against rabbit (1:20,000, A0545, Sigma) or mouse (1:20,000, A2554, Sigma) linked to horseradish peroxidase. Signals were visualized by chemiluminescence (ECL, Elabscience) on autoradiographic film (Fujifilm X-Ray Film).

## 4.4 Angelman Syndrome animal model

### *4.4.1 Animals*

Ube3Atm1Alb/J mice, used as an animal model of Angelman syndrome (AS mice), were purchased from The Jackson Laboratory (Bar Harbor, MN). Wild-type (WT) and AS mice were obtained in-house through breeding of heterozygous females with WT males. Genotype was determined as previously reported (Baudry et al.,2012). Animal handling and experimental usage were performed following protocols approved by the Western University of Health Sciences (Pomona,California) Institutional Animal Care and Use Committee (IACUC), in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Male and female AS mice ranging from 2 to 4 months of age and their littermate WT mice as controls, matched in sex and age, were used. Animals were housed in groups of two to three per cage and exposed to a 12-h light/dark cycle with food and water available ad libitum (Baudry et al 2012).

### *4.4.2 Electrophysiology in acute hippocampal slices*

Acute hippocampal transversal slices (350 µm-thick) were prepared from 3 months old mice according to standard methods (Kramar et al 2006). Slices were maintained in an interface recording chamber under continuous perfusion with oxygenated (95% $O_2$/5% $CO_2$) and preheated (33 ± 0.5 °C) artificial cerebrospinal fluid (aCSF) containing 110 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM $MgSO_4$, 1.24 mM $KH_2PO_4$, 10 mM D-glucose, 27.4 mM $NaHCO_3$. Field excitatory postsynaptic potentials (fEPSPs) were recorded in CA1 stratum radiatum following stimulation of the Schaffer collateral pathway as previously described (Baudry et al 2012). Long-term potentiation was induced using theta burst stimulation (TBS; 10 bursts at 5 Hz, with each burst made by 4 pulses delivered at 100 Hz, with an interburst interval of 200 ms). Amplitudes and slopes of fEPSPs were measured and normalized to the average of the values recorded during the 10-min baseline before TBS. For each mouse brain, one or two slices were analyzed. Data were collected and digitized by Clampex.

### *4.4.3 Fear conditioning*

AS and WT mice were randomly assigned to either LP-211 or vehicle groups and blinded to the examiner. LP-211 was dissolved in a vehicle solution (0.5% DMSO in 0.9% NaCl). AS and WT mice assigned to drug or vehicle group received intraperitoneal (ip) injections of LP-211 (3 mg/kg) or vehicle solution respectively, 30 min before the training session, as previously reported (Costa et al 2018).

Mice were handled daily for 5 days prior to training. The fear-conditioning test was performed during the last 6 h of the light cycle. The conditioning chamber, a Plexiglas cage (29 cm×29 cm×29 cm) with a grid floor made by stainless steel rods (Coulbourn Instruments, Allentown, PA), was

placed in a sound-attenuating box. The conditioning chamber was cleaned with 10% ethanol to provide a background odour. A ventilation fan provided a background noise at about 55 dB. The animal behaviour was recorded by a video camera system. On the first day of training, mice were placed in the chamber and after a 2 min exploration period, three tone-foot-shock pairings separated by 1 min intervals were delivered. The tone (85 dB 2 kHz) lasted for 30 s, and, in the last 2 s, the foot-shock (0.75 mA) was administered. The inter-trials intervals lasted 1 min. Mice remained in the training chamber for an additional 30 s after the last foot shock and then they were returned to their home cages. Twenty-four hours after training, the context test was performed. Mice were placed back into the conditioning chamber, without foot shocks or tones, and their behaviours were recorded for 5 min. Forty-eight hours after training, animals were subjected to the cue/tone test. To perform this test, the conditioning chamber was modified by replacing the metal grid floor with a plastic sheet, white metal walls with plastic walls gridded with red tapes, and ethanol odour with acetic acid. The ventilation fan was turned off to reduce the background noise and the ceiling light was changed from yellow to white. Mice were placed in the altered chamber for an acclimatation period of 5 min during which their freezing level in the altered context was measured. After this period, a tone (85 dB, 2 kHz) was delivered for 1 min to measure their freezing level in response to tone. Mice behaviour was recorded with the Freezeframe software and analysed with the Freezeview software (Coulbourn Instruments). Freezing was defined as the absence of all visible non-respiratory movements of the body and vibrissae, for more than 1 s. The percent of time animal froze was calculated and the group means with SEM and accumulative distribution of % freezing was analysed (Sun et al., 2015a).

### 4.5 Cerebral Organoids

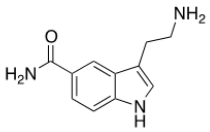
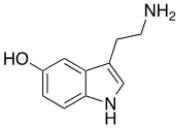
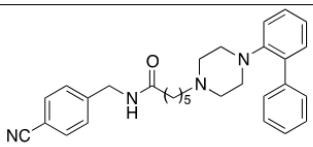
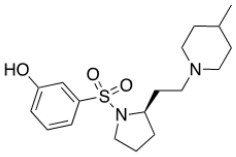
Human cerebral organoids (hCO) were prepared by Prof. Rossella Di Giaimo. Briefly, induced pluripotent stem cells reprogrammed from human newborn foreskin fibroblasts (CRL-2522, ATCC) (O'Neil et al., 2018; Klaus et al., 2019) were used to generate cerebral organoids as previously described (Lancaster et al., 2013; Lancaster and Knoblich, 2014). Organoids were kept in 10 cm dishes on an orbital shaker at 37 °C, 5% $CO_2$ and ambient oxygen level with medium changes every 3 to 4 days. Organoids were analyzed 40 days after plating. For synaptosomal fraction purification, a pool of 20-40 organoids was collected by centrifugation (500 *g* for 10 min). Sedimented organoids were homogenized in a Dounce homogenizer with 9 volumes of HM. The P2 crude synaptosomal fraction was prepared as described before. Homogenate and P2 fraction, resuspended in sample buffer, were processed for western blot analysis as previously described.

# 5. RESULTS

## 5.1 Binding kinetics: initial setting using a 5-HT7R radioligand

Binding kinetics of a ligand to a GPCR can be assessed using different methodologies, including radioligand binding assay and fluorescence-based methods (Sykes et al 2019). In this study, radioligand binding assays were performed using [$^3$H]-5-CT, a radiolabeled agonist commonly used to assess 5-HT7R affinity, and membrane preparations from HEK-293 cell stably transfected with human 5-HT7R. Initial experiments were aimed at fully characterizing [$^3$H]-5-CT binding kinetic parameters because available data were incomplete or different experimental protocols were used (Satala et al 2018; Armstrong et al 2020). [$^3$H]-5-CT equilibrium and kinetic parameters are reported in Table 3. The $k_{on}$ and $k_{off}$ were determined as detailed in Material and Methods. The $k_{on}$ was obtained from the association curve (Figure 3A). To determine $k_{off}$, 5-HT7Rs were pre-labelled with a [$^3$H]-5-CT concentration (approximately 10 × $K_d$) able to provide high initial receptor occupancy. Then, radioligand dissociation was induced by addition of a saturating concentration of unlabeled 5-CT (approximately 1000 × $K_d$; Figure 3B). The kinetically derived $K_d$ for [$^3$H]-5-CT (kinetic $K_d = k_{off}/k_{on}$, 0.18 nM) was in good agreement with the $K_d$ obtained from saturation experiments ($K_d$ = 0.20 nM) (Table 3).

**Table 3. Equilibrium and kinetic binding parameters of reference compounds**

| Compd | | Equilibrium $K_i$ ± SEM (nM) | $k_{on}$ ± SEM ($M^{-1}$, $min^{-1}$) | $k_{off}$ ± SEM ($min^{-1}$) | Kinetically derived $K_d = k_{off}/k_{on}$ ± SEM (nM) | RT = $1/k_{off}$ ± SEM (min) |
|---|---|---|---|---|---|---|
| [$^3$H]-5-CT | | 0.20 ± 0.05 | (9.350±3.12)×$10^7$ | 0.01679±0.0061 | 0.18 ± 0.06 | 59 ± 13 |
| 5-CT |  | 0.43 ± 0.13 | (3.140±1.13)×$10^7$ | 0.01976±0.0059 | 0.63 ± 0.17 | 50 ± 12 |
| 5-HT |  | 1.20 ± 0.31 | (2.364±0.12)×$10^8$ | 0.2759±0.0138 | 1.16 ± 0.40 | 3.6 ± 0.2 |
| LP-211 |  | 20.0 ± 3.2 | (1.744±0.23)×$10^6$ | 0.04157±0.0051 | 23.8 ± 4.2 | 24.0 ± 2.9 |
| SB-269970 |  | 3.50 ± 0.81 | (2.641±0.70)×$10^7$ | 0.08221±0.0205 | 3.11 ± 0.78 | 12.0 ± 3.1 |

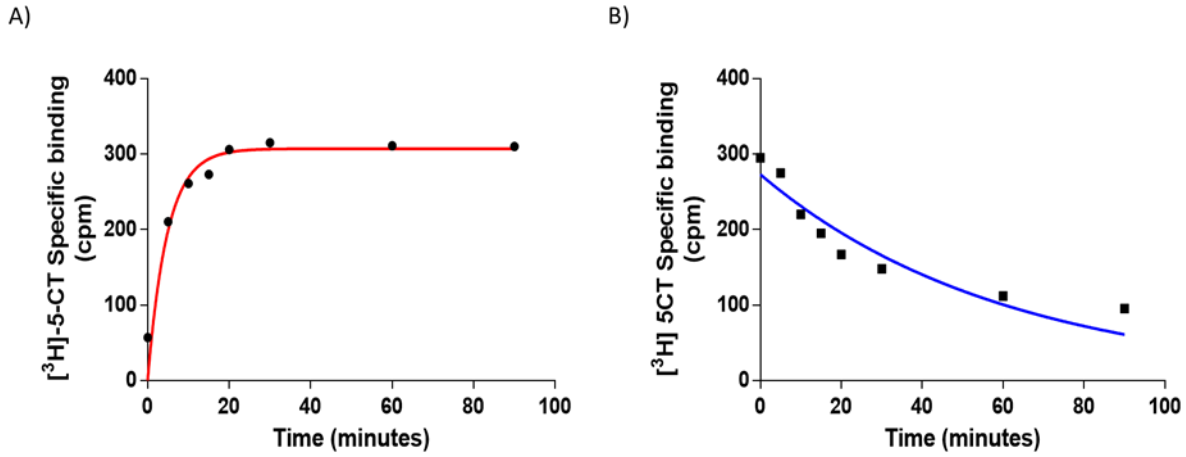



**Figure 3.** [$^3$H]-5-CT binding kinetics. A) [$^3$H]-5-CT association kinetics. B) [$^3$H]-5-CT binding dissociation kinetics

### *5.1.1 Binding kinetics of 5-HT7R ligands*

Considering the paucity of data in the literature on the binding kinetics of 5-HT7R ligands, once we determined the $k_{on}$ and $k_{off}$ of [$^3$H]-5-CT, we assessed the kinetic parameters and residence time of several 5-HT7R reference agonists and antagonists (Table 4). First, we measured affinity values (Ki) of various 5-HT7R ligands by competitive binding assay as reported in Materials and Methods (Figure 4). Then, a concentration of the unlabeled competitor (approximately 10 × $K_i$) was added simultaneously with the radioligand to the receptor and the experimentally derived rate of specific radioligand binding was analyzed using the equations developed by Motulsky and Mahan (Motulsky and Mahan 1984). This approach allowed to determine the association and dissociation rates of each reference compound ($k_{on}$ and $k_{off}$) that were used to calculate their kinetic $K_d$ and residence time (Table 4).

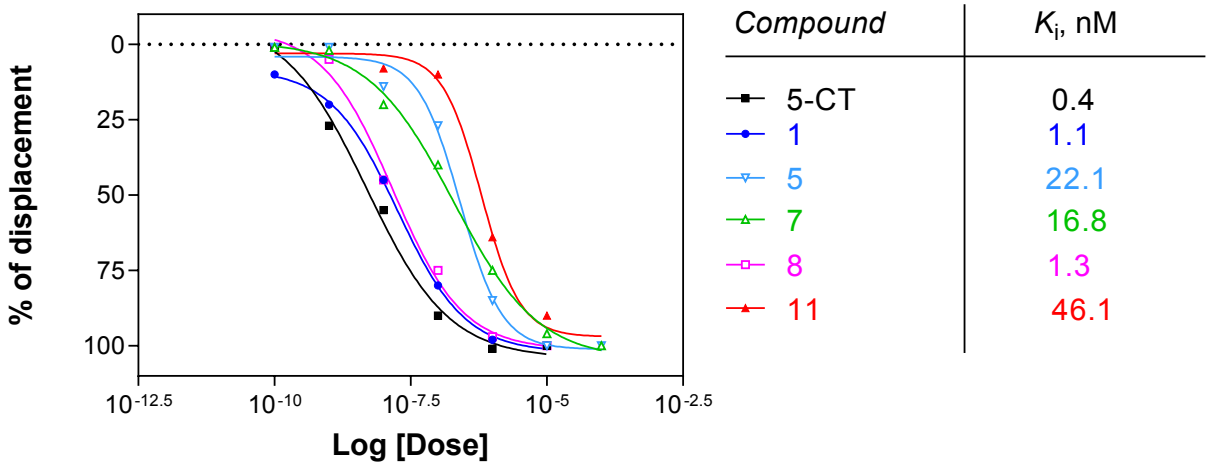
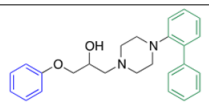
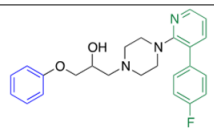
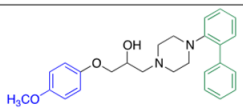
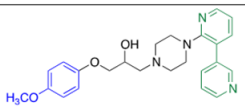
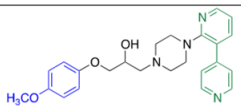
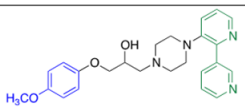
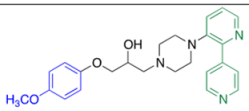
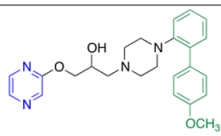
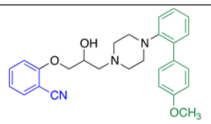
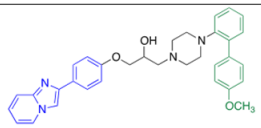
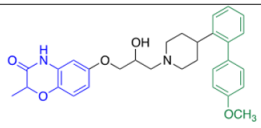
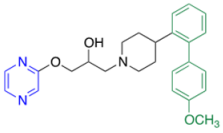



**Figure 4**. Examples of competitive binding curve for some of the 5-HT7R compounds. The displacement curves were obtained by plotting the percentage of displacement as a function of the logarithmic value of the dose.

Once the method to evaluate binding kinetics of 5-HT7R ligands was established, we extended the analysis to a selected set of arylpiperazine-based 5-HT7R ligands belonging to the in-house library of Marcello Leopoldo laboratory and characterized by the general formula Ar–piperazine–aryloxypropanol linker–terminal fragment. It has been proposed that general physicochemical properties of a ligand, such as lipophilicity or rotational bonds, may affect the residence time and that modulating such properties can lead to "fine-tuned drug-target binding kinetics" (Pan et al 2013, Tresadern et al 2011, Vilums et al 2015, Soethoudt et al 2018). Thus, to address this aspect, we selected a set of compounds covering a wide range of lipophilicity (expressed as $clogD_{7.4}$), from $clogD_{7.4}$ = 4.13 for the most lipophilic compound (**1**) to $clogD_{7.4}$ = 1.78 for the less lipophilic compound (**11**) (Hansen et al 2014, Lacivita et al 2020). The set also included 5-HT7R ligands having similar $clogD_{7.4}$ values but variable lipophilicity of their Ar group or terminal fragment. Thus, we assessed the binding kinetic parameters of compounds **1-12** to check whether the overall lipophilicity of the molecule or the lipophilicity of a specific fragment could affect the kinetics of drug-receptor interaction (Table 4).

**Table 4.** Equilibrium and kinetic binding parameters of the selected set of 5-HT7R ligands. [a]Calculated with ChemAxon Software (Instant JChem 15.3.30.0, ChemAxon, 2015. http://www.chemaxon.com.); [b]Data taken from ref. Hansen et al 2014; [c]Data taken from Lacivita et al 2020

| Compd | | ClogD$_{7.4}$[a] | Equilibrium $K_i$ ± SEM (nM) | $k_{on}$ ± SEM ($M^{-1}$, $min^{-1}$) | $k_{off}$ ± SEM ($min^{-1}$) | Kinetically derived $K_d$= $k_{off}/k_{on}$ ± SEM (nM) | RT = $1/k_{off}$ ± SEM (min) |
|---|---|---|---|---|---|---|---|
| **1** |  | 4.13 | 1.31 ± 0.20[b] | (1.817±0.06) ×10$^7$ | 0.02154±0.0073 | 1.18 ± 0.30 | 46.0 ±15.2 |
| **2** |  | 3.81 | 47.5 ± 3.5 | (2.764±0.64) ×10$^6$ | 0.1405±0.0285 | 50.8 ± 4.5 | 7.0 ±1.4 |
| **3** |  | 3.99 | 15.0 ± 1.8 | (2.417±0.33)×10$^6$ | 0.03235±0.0045 | 13.4 ± 2.1 | 31.0 ±4.3 |
| **4** |  | 2.30 | 19.3 ± 1.1[b] | (4.708±1.23) ×10$^6$ | 0.1145±0.0274 | 24.3 ± 3.1 | 8.7 ± 2.1 |
| **5** |  | 2.29 | 22.1 ± 0.1[b] | (4.581±0.91) ×10$^6$ | 0.1178±0.0234 | 25.7 ± 1.1 | 8.5 ± 1.7 |
| **6** |  | 2.03 | 57.0 ± 1.7[b] | (4.592±0.95) ×10$^5$ | 0.03012±0.0084 | 65.6 ± 3.8 | 33.0 ± 9.2 |
| **7** |  | 2.09 | 16.8 ± 3.5[b] | (2.804±0.25) ×10$^6$ | 0.06997±0.0282 | 24.9 ± 1.7 | 16.0 ± 9.3 |
| **8** |  | 2.28 | 1.10 ± 0.30[b] | (3.984±0.43) ×10$^7$ | 0.05045±0.0027 | 1.27 ± 0.30 | 19.8 ± 1.1 |
| **9** |  | 3.88 | 17.7 ± 5.0[c] | (1.878±0.24) ×10$^6$ | 0.03343±0.0011 | 17.8 ± 2.5 | 30.0 ± 0.9 |
| **10** |  | 4.0 | 25.7 ± 2.6[c] | (1.491±0.24) ×10$^6$ | 0.04379±0.0150 | 29.4 ± 5.1 | 22.8 ± 7.7 |
| **11** |  | 1.78 | 46.7 ± 3.7 | (1.759±0.08) ×10$^6$ | 0.07455±0.0160 | 42.4 ± 6.2 | 13.0 ± 2.7 |
| **12** |  | 1.87 | 15.2 ± 1.4[c] | (1.545±0.54) ×10$^6$ | 0.02915±0.0093 | 18.9 ± 2.3 | 34.0 ± 1.1 |

The kinetic constants ($K_d$) of all compounds were then compared to their equilibrium affinities ($K_i$). A statistically significant correlation was found between the negative logarithm of the kinetic $K_d$ ($pK_d$) and the equilibrium $pK_i$, indicating that the method produced accurate $k_{on}$ and $k_{off}$ rates (Figure 5A). Moreover, a linear correlation was also found between $pK_d$ and $k_{on}$ (Figure 5B), but not between $pK_d$ and $k_{off}$ (Figure 5C), suggesting that the binding affinity was mostly influenced by the association rate rather than the dissociation rate, as reported for other GPCRs (Sykes et al 2012; Yu et al 2015; Liu et al 2020).

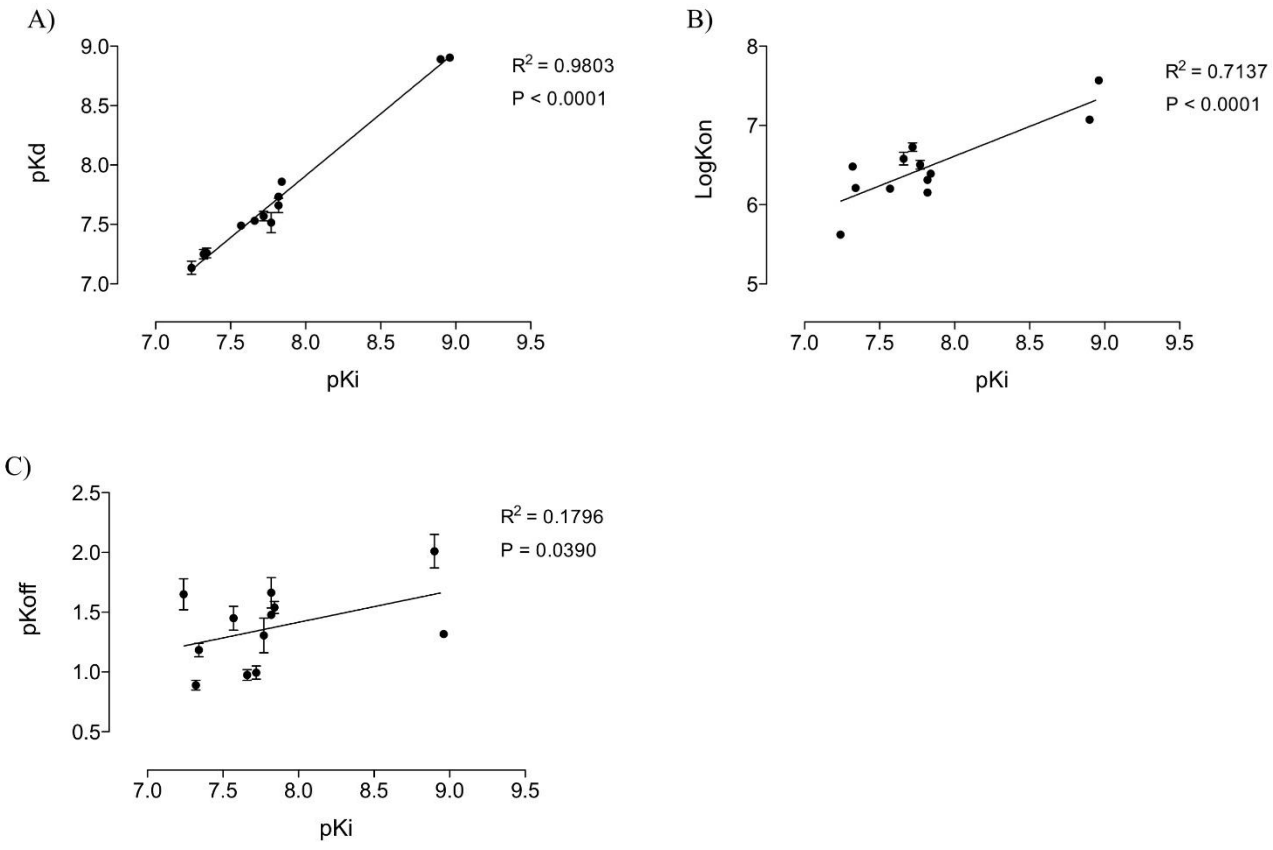



**Figure 5.** Comparison between equilibrium binding affinity and binding kinetics. Correlation plots of equilibrium affinity (pKi) with (A) negative logarithmic transformation of kinetic affinity ($pK_d$), (B) association rate $k_{on}$, and (C) dissociation rate $k_{off}$.

The kinetic profile of the compounds enabled to describe structure-kinetics relationships. The highest residence time was shown by compound **1**, which was also the most lipophilic compound of the series. The lowest residence times were shown by compounds **2**, **4**, and **5**. It is noteworthy that compounds **4** and **5** have very similar lipophilicity (and residence time), whereas compound **2** has a $clogD_{7.4}$ value 1.5 log unit higher than **4** and **5**. This clearly suggests that the overall lipophilicity of the molecule is not the main property that influences the residence time. Indeed, no correlation was found between the $k_{on}$, $k_{off}$, or residence time and $clogD_{7.4}$ values (data not shown). Comparing compounds **4** and **5** (featuring a 2-pyridyl group linked to the piperazine ring) with the corresponding 3-pyridyl isomers **6** and **7**, it can be noted that the simple formal shifting of the pyridine aza group influences the residence time. In fact, the residence times of compounds **6** and **7** are 4- and 2-fold higher than the residence time of compounds **4** and **5**, respectively. These data suggest that the position of the polar aza group in the biphenyl-like system linked to the piperazine ring has a relevant role on the kinetics of ligand-5-HT7R interaction.

Compounds **3**, **8**, **9**, **10**, and **12**, which have no polar groups on the biphenyl system and present terminal groups characterized with different lipophilic properties, had residence times higher than 20 min. These data confirmed that the overall lipophilicity of the molecule was not correlated with the residence time. In fact, compounds **3** and **12** showed very similar residence time and 2-units difference in $clogD_{7.4}$ value.

Finally, we tested compound **11** which features the 2-acetylphenyl ring linked to the piperazine ring instead of the 4-methoxybiphenyl system. We found that the residence time of **11** was very close to that of compounds **4** and **5**, suggesting that the lipophilicity of the ring system linked to the piperazine ring has a role in the residence time of this group of compounds. In fact, the variation

2-acetylphenyl/4-methoxybiphenyl leads to a reduction of lipophilicity (ΔClogD7.4 = 1.56) similar to that of the variation bipyridyl/4-methoxybiphenyl (ΔClogD7.4 = 1.53).

Collectively, the data suggest that the lipophilicity of the "right hand" part compared to the "left hand" part of the molecule has a greater impact on the kinetics of the interaction between the ligand and 5-HT7R. In addition, the data suggest that the position of polar groups in the "right hand" part of the molecule has an impact on the residence time.

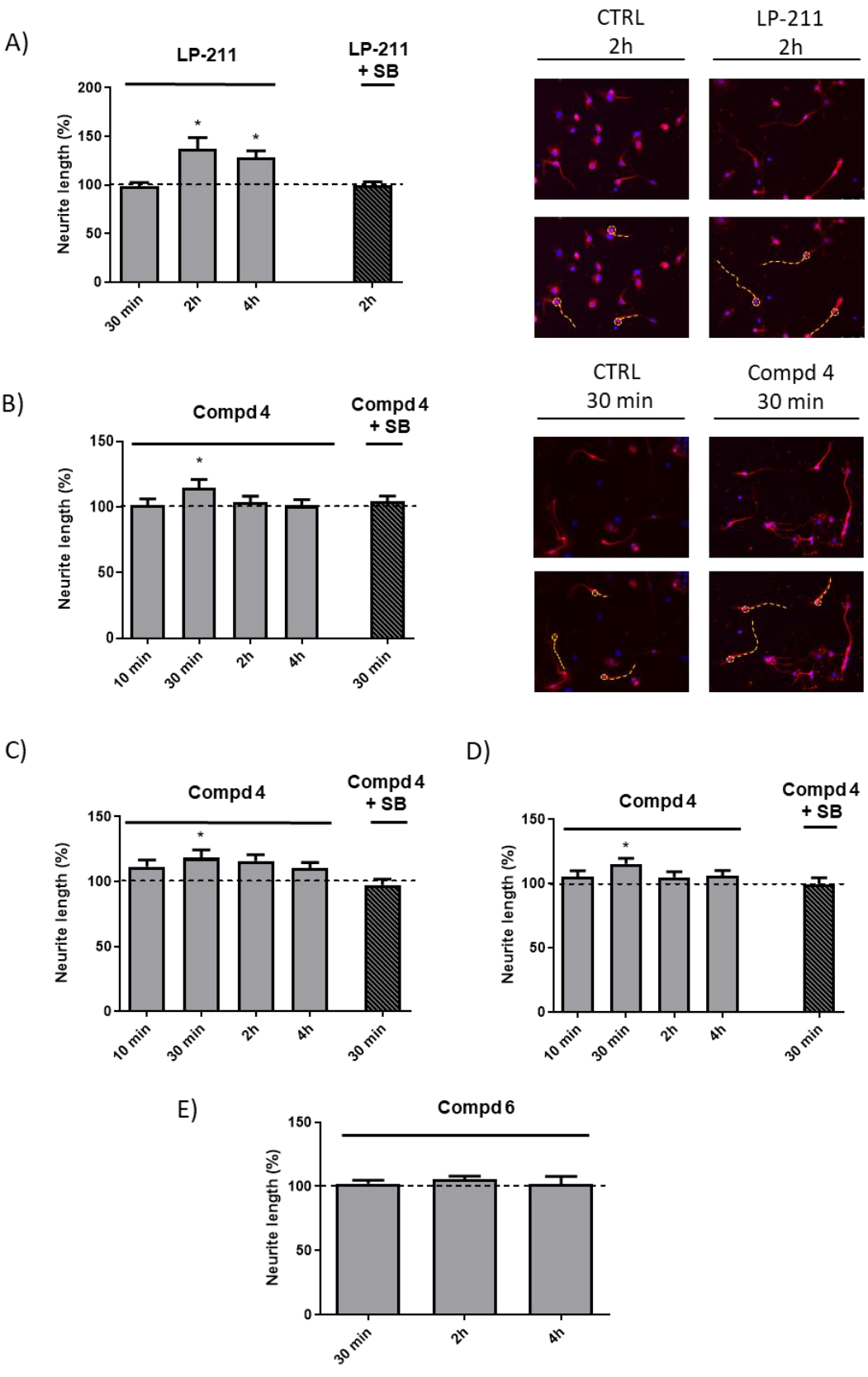
**5.2 Effect of 5-HT7R agonists on neurite outgrowth**

We previously reported that pharmacological stimulation of 5-HT7R with the selective agonist LP-211 in rodent primary neurons in culture significantly increased neurite outgrowth, as compared to vehicle-treated control cultures (Speranza et al 2013, 2015, 2017). An interesting aspect of LP-211 action was that its effect started after 2 h of stimulation and was still present after 4 h, then progressively diminished over time (Speranza et al 2013, 2015, 2017, Volpicelli et al 2019). This effect was 5-HT7R-specific, as neurite elongation was not observed in neurons treated with LP-211 and the selective 5-HT7R antagonist SB-269970.

The analysis of the arylpiperazine derivatives reported in Table 4 led to the identification of compounds **2**, **4**, and **5,** which have residence times close to 8 min, i.e.:., 3-fold lower than LP-211, which has a residence time of 24 min. Thus, in an initial attempt to correlate the residence time with biological activity, we selected compound **4**, which shows a $K_i$ value very close to that of LP-211 and evaluated its effect on neurite elongation, as compared to LP-211. After 3 days in culture, neuronal primary cultures dissociated from striatum of postnatal day 1 (P1) or 3 (P3) mice

were stimulated for 10 min, 30 min, 2 h and 4 h with LP-211 in presence or absence of the specific 5-HT7R antagonist SB-269970 (Figure 6 A). In parallel, the same neuronal cultures dissociated from striatum, cortex and hippocampus of P1-P3 mice were stimulated with compound **4** in presence or absence of SB-269970 (Figure 6 B-D). As expected, stimulation of striatal cultures with LP-211 resulted in increased neurite outgrowth, as compared to vehicle treatment, starting at 2h and still present at 4h incubation. The effect selectively depended on the stimulation of 5-HT7R as it was reversed by the specific antagonist SB-269970 (Figure 6A). Similarly, stimulation of striatal cultures with 100 nM of compound **4** induced a time-dependent increase in neurite length, as compared to control, but the peak effect was at 30 min (about 15% increase, Figure 6 B). Although neurite length still appeared higher than control at 2 h, the increase was not statistically significant. Similar results were also obtained in cortical and hippocampal neurons (Figure 6 C-D). This morphogenic effect was completely abolished when compound **4** was incubated in the presence of SB-269970 (Figure 6 B), demonstrating that the increased neurite length was specifically due to the selective stimulation of 5-HT7R by compound **4**. Interestingly, the effect of compound **4** displayed a different timing when compared to LP-211, as it starts much earlier (30 min) and ends rapidly (Figure 6). In addition, compound 4 increased the neurite length only of 15%, while the effect of LP-211 was more robust reaching about 40%. We also observed the effect of another compound using neuronal primary cultures prepared from mouse embryos. In particular we used compound **6**, which has a residence time close to 33 min, i.e., 1.5 -fold higher than LP-211. Hippocampal primary cultures from E18 mice, incubated with compound 6 up to 4 h, did not shown any significant modification in neurite length (Figure 6 E).

**Figure 6. Effect of 5-HT7R ligands on neurite outgrowth in neuronal primary cultures**. Striatal primary neurons from P1-P3 mice were treated with A) LP-211 (100 nM) or B) compound **4** (compd **4**; 100 nM) at different time points alone or in presence of the selective 5-HT7R antagonist SB-269970 (100 nM).LP-211 improve neurite length of about 40%. The panels on the right display representative images of CTRL and drug-treated neurons immunostained with the neuronal marker Tuj1 (red) and counterstained with the nuclear marker DAPI (blue; magnification 20x). The dashed yellow lines are manually drawn by the operator from the soma (yellow circle) to the end of the primary neurite in order to measure neurite length. C) Cortical and D) hippocampal primary neurons from P1-P3 mice treated with compound **4** (100 nM) at different time points. E) Hippocampal primary neurons from E18 mice were treated with compound **6** (compd **6**) (100 nM) at different time points. Neurite length was expressed as the percentage of the values measured in the corresponding vehicle-treated cultures (CTRL, set to 100%, dashed line). The bars represent means ± SEM from randomly selected fields for each cell culture condition (n= 4). * Significantly different from CTRL by Student's t-test ($p < 0.05$).


A)
LP-211
LP-211 + SB
Neurite length (%)
200
150
100
50
0
30 min
2h
4h
2h
CTRL 2h
LP-211 2h
B)
Compd 4
Compd 4 + SB
Neurite length (%)
150
100
50
0
10 min
30 min
2h
4h
30 min
CTRL 30 min
Compd 4 30 min
C)
Compd 4
Compd 4 + SB
Neurite length (%)
150
100
50
0
10 min
30 min
2h
4h
30 min
D)
Compd 4
Compd 4 + SB
Neurite length (%)
150
100
50
0
10 min
30 min
2h
4h
30 min
E)
Compd 6
Neurite length (%)
150
100
50
0
30 min
2h
4h

## 5.3 Effect of 5-HT7R activation by LP-211 on synaptosomes from rat brain

### *5.3.1 Characterization of synaptosomal fraction*

To further analyse the role of 5-HT7R in the physiology of the nervous system and in particular in synaptic plasticity, the effects of stimulation of 5-HT7R by LP-211 were studied in synaptosomal fractions isolated from rat cerebral cortex.

Under basal conditions, the level of the presynaptic proteins synaptophysin and synaptotagmin was significantly higher in synaptosomes, as compared to homogenates, as expected for a fraction enriched in presynaptic compartments (Figure 7 A-C, E). 5-HT7R was present in synaptosomes and its expression level in this fraction was not significantly different from the corresponding homogenate (Figure 7 A,D,E), indicating that the receptor does not have a preferential localization in presynaptic terminals.

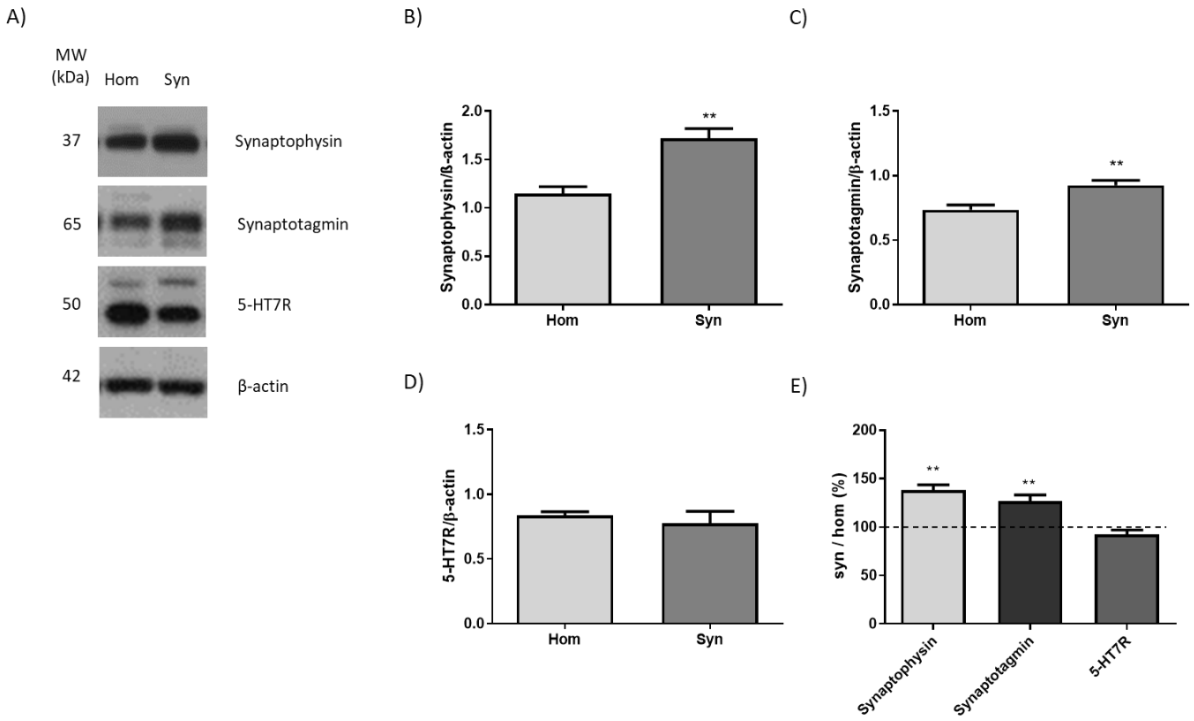


**Figure 7**. **Expression level of synaptophysin, synaptotagmin and 5-HT7R in synaptosomes and in the corresponding homogenates.** A) Representative images of Western Blots on synaptosomes and homogenates, using antibodies against synaptophysin, synaptotagmin, 5-HT7R and β-actin. Relative intensity of (B) synaptophysin, (C) synaptotagmin and (D) 5-HT7R normalized to β-actin in homogenates and synaptosomes. E) Enrichment of expression levels of the three proteins in synaptosomes versus homogenates. Levels in homogenates were set at 100% (dashed line). Data are means ± SEM of n = 5. T-test for paired data. The asterisks indicate values significantly different from the ones in homogenates (** p value <0.01). Hom: homogenate; Syn: synaptosomes; MW: molecular weight; kDa: kDalton.

### *5.3.2 Activation of synaptosomal 5-HT7R leads to ERK phosphorylation*

Incubation of synaptosomal fractions with LP-211 resulted in a significant increase in ERK phosphorylation (Figure 8 A-B, LP-211 versus vehicle). This is in agreement with literature data indicating activation of the ERK pathway in response to stimulation of 5-HT7R by LP-211 in primary neuronal cultures (Speranza et al. 2013, 2015). Therefore, synaptosomes can be used as an experimental model to investigate the involvement of 5-HT7R in nerve ending physiology.

Interestingly, when synaptosomes were incubated for 2 h, even without LP-211, a significant increase in ERK phosphorylation was observed, as compared to non-incubated fractions (Figure 8 A-B vehicle versus syn), suggesting that the ERK pathway is already activated by synaptosome incubation, independently of 5-HT7R, and its activation is potentiated by LP-211. It is possible to hypothesize that ERK phosphorylation is linked to local protein synthesis, which is known to be activated under these incubation conditions (Eyman et al 07, 13, Ferrara et al 09).

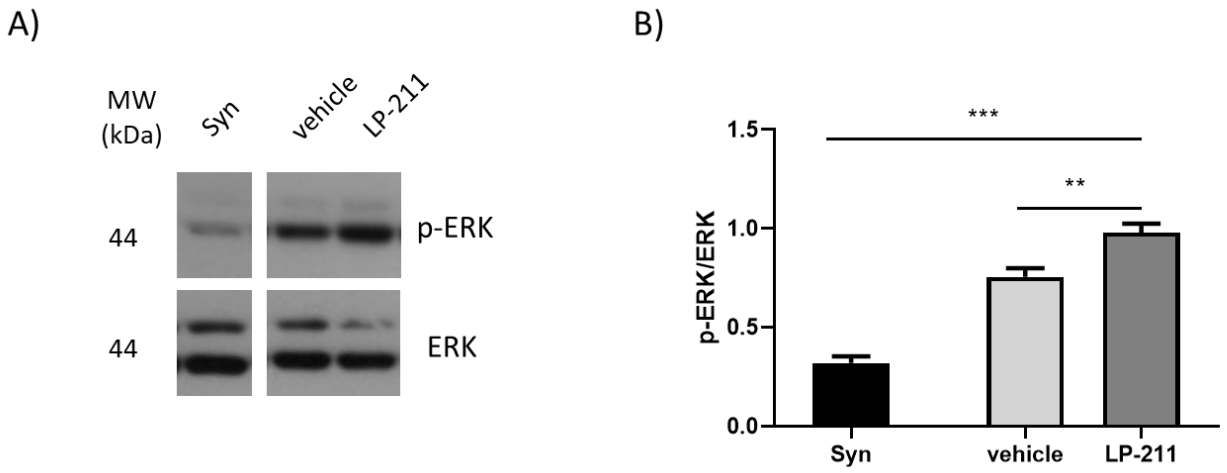



**Figure 8: Activation of the ERK signaling pathway following the incubation of synaptosomes with LP-211**. A) Representative images of Western Blot analysis on synaptosomes non-incubated or incubated with or without LP-211, using antibodies against ERK and pERK. B) Relative intensity of p-ERK normalized to ERK in non-incubated synaptosomes (syn) or synaptosomes incubated for 2h in the presence of 100 nM LP-211 or vehicle (DMSO). Data are means ± SEM of n=4. T-test for paired data. **p value <0.01, ***p value <0.001. MW: molecular weight; kDa: kDalton.

### *5.3.3 Stimulation of synaptosomal 5-HT7R is not linked to mTOR pathways*

Stimulation of 5-HT7R with LP-211 in neuronal primary cultures from embryonic mouse brain selectively activates mTORC1, leading to cytoskeleton reorganization and consequent axonal elongation, increased formation of dendritic spines and facilitated synaptogenesis (Speranza et al 2015,2017). Downstream effectors of mTORC1, including the p70/ribosomal S6 kinase (p70/S6K), regulate RNA (mRNA) translation, protein synthesis, lipogenesis and energy metabolism, while activation of mTORC2 is involved in actin cytoskeleton dynamics and cell survival mainly through Akt (Laplante and Sabatini, 2012).

Thus, we investigated the possible effect of 5-HT7R stimulation in synaptosomes on mTOR pathways. After incubation of synaptosomes with LP-211, no significant increase in P70 or AKT phosphorylation levels was observed (Figure 9 A-B, D). However, it is interesting to notice that in synaptosomes incubated for 2 h (with or without LP-211), a significant increase of P70 phosphorylation was observed, as compared to non-incubated fractions (Figure 9 B, vehicle versus syn), while a significant decrease of Akt phosphorylation was observed (Figure 9 D, vehicle versus syn). These data indicate that activation of mTORC1 in synaptosomes is independent from 5-HT7R. Thus, it is possible to hypothesize that activation of local protein synthesis in synaptosomes (Eyman et al 07, 13, Ferrara et al 09), depends on TORC1 activation.

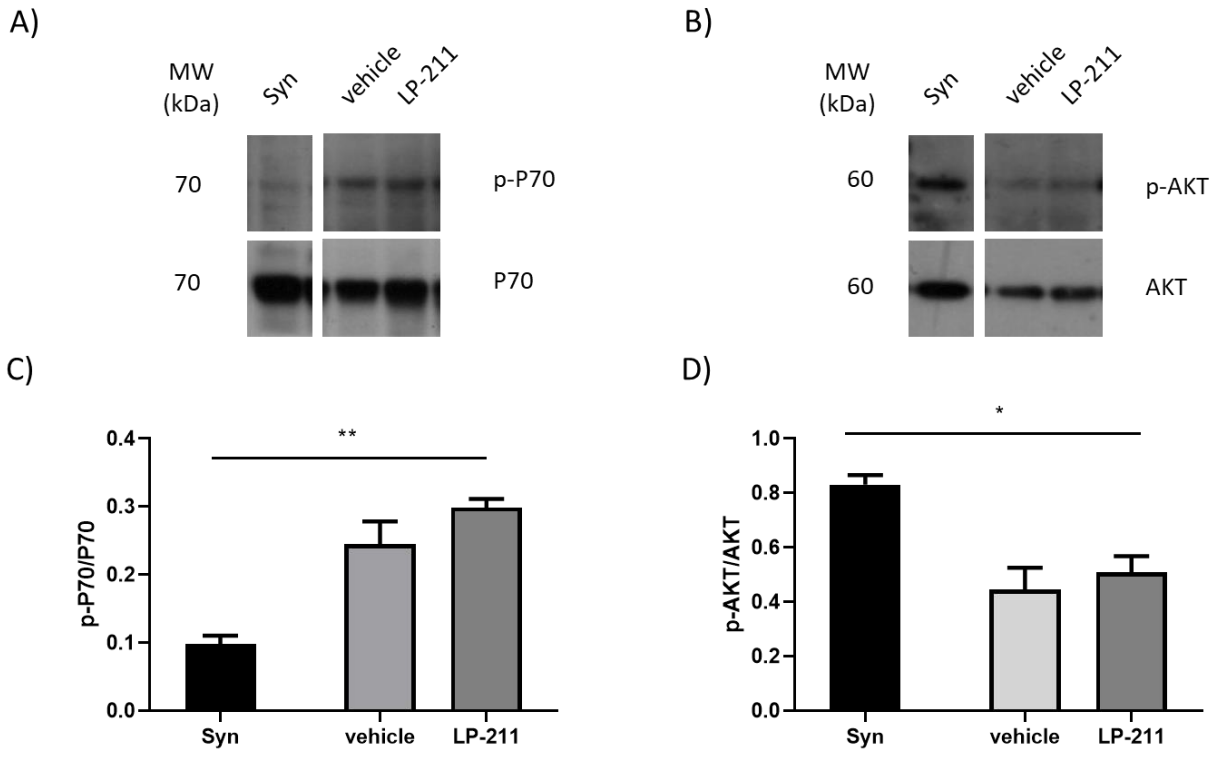


**Figure 9**: **Effect of LP-211 on mTOR signaling pathways.** A-B) Representative images of Western Blot analysis on synaptosomes non-incubated, or incubated with or without LP-211, using antibodies against P70, pP70, AKT and pAKT. C) Relative intensity of p-P70 normalized to P70 in non-incubated synaptosomes (syn) or synaptosomes incubated for 2h in the presence of 100 nM LP-211 or vehicle (DMSO). D) Relative intensity of p-AKT normalized to AKT in non-incubated synaptosomes (syn) or synaptosomes incubated for 2h in the presence of 100 nM LP-211 or vehicle (DMSO). Data are means ± SEM of n=4. T-test for paired data. * p value <0.05, ** p value <0.001. MW: molecular weight; kDa: kDalton.

### *5.3.4 Effect of 5-HT7R activation on synaptosomal expression level of eukaryotic initiation factors*

It was previously demonstrated that neurite elongation mediated by LP-211 activation of 5-HT7R requires newly synthesized proteins (Speranza et al. 2013).

To verify the possible effect of LP-211 treatment on synaptosomal protein synthesis, a preliminary experiment was designed to investigate the modulation of expression levels of the initiation factor of eukaryotic protein synthesis (eIF2B2) in synaptosomes incubated with or without LP-211. This factor is locally synthesized in axons of rat superior cervical ganglia neurons (Kar et al 2013), and its axonal downregulation was demonstrated to inhibit local protein synthesis (Kar et al., 2013).

eIF2B2 was present in the synaptosomal fraction and its expression level surprisingly decreased when synaptosomes were incubated with LP-211 (Figure 10 A-B, E). On the other hand, expression levels of the cytoskeletal proteins β-actin and α-tubulin were not affected by LP-211 (Figure 10 A, C-D); thus, each of them can be used to normalize the expression level of eIF2B2

(Figure 10 E). These results suggested that stimulation of 5-HT7R leads to the inhibition of synaptosomal protein synthesis in the whole cortex.

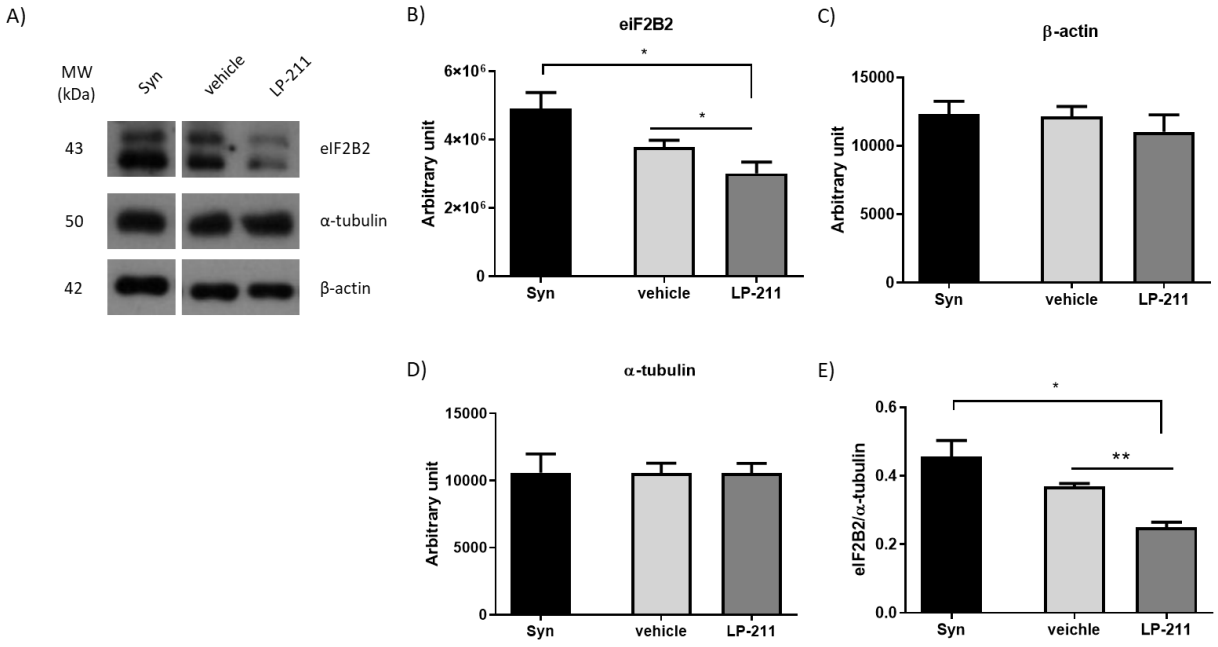



**Figure 10: LP-211 modulates the expression levels of the eukaryotic initiation factors eIF2B2 in the synaptosomes.** A) Representative images of the Western Blot analysis on synaptosomes non-incubated or incubated with or without LP-211. Expression levels of B) eIF2B2**,** C**)** β-actin and D) α-tubulin) in non-incubated synaptosomes (syn) or synaptosomes incubated for 2h with 100 nM LP-211 or vehicle (DMSO). E) Relative intensity of eIF2B2 normalized to α-tubulin in non-incubated synaptosomes (syn) or synaptosomes incubated for 2h with 100 nM LP-211 or vehicle (DMSO). Data are means ± SEM of n=4. T-test for paired data. * p value <0.05 ** p value <0.01. MW: molecular weight; kDa: kDalton.

### *5.3.5 Effect of 5-HT7R activation on cystatin B turnover in synaptosomes*

Cystatin-B, a protein belonging to the protease inhibitor family, has been previously demonstrated to be locally synthetized in synaptosomes and secreted by depolarization (Penna et al 2019). This result was confirmed by experiments showing that expression levels of cystatin B significantly increased in synaptosomes incubated for 2 h (Figure 11).

When the incubation was carried out in the presence of LP-211, expression levels of cystatin B significantly decreased, suggesting that stimulation of 5-HT7R was affecting the turnover of this protein in synaptic compartments (Figure 11). This response is specific for cystatin B, since expression levels of other proteins such as α-tubulin were not affected by LP-211.

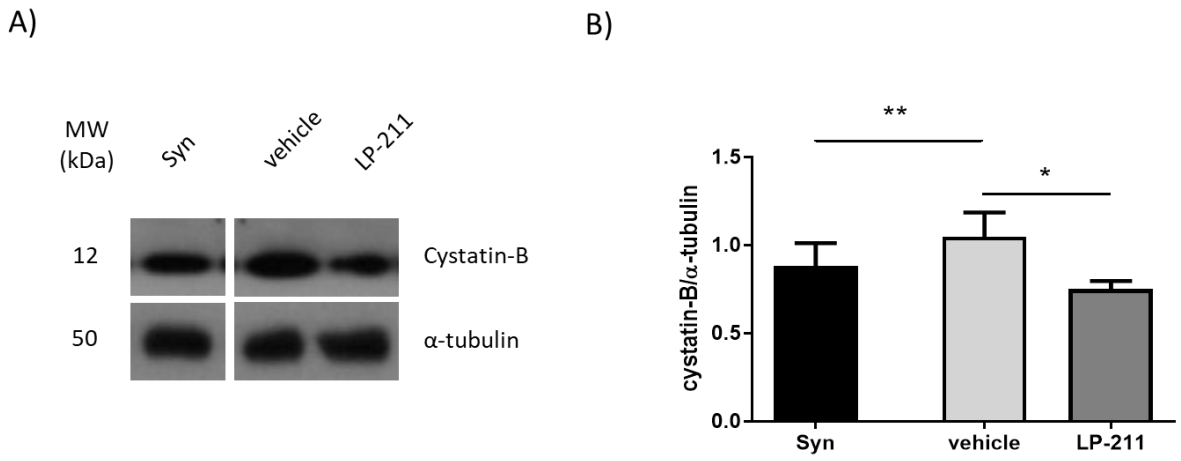



**Figure 11**: **LP-211 modulates the expression levels of cystatin-B in the synaptosomes.** A) Representative images of the Western Blot analysis on synaptosomes non-incubated, or incubated with or without LP-211, using antibodies against cystatin B and α-tubulin. B) Relative intensity of cystatin-B normalized to α-tubulin in non-incubated synaptosomes (syn) or synaptosomes incubated for 2h in the presence of 100 nM LP-211 or vehicle (DMSO). Data are means ± SEM of n=4. T-test for paired data. * p value <0.05, ** p value <0.01. MW: molecular weight; kDa: kDalton.

Since cystatin B has been demonstrated to be secreted by synaptosomes, we also verified whether 5-HT7R stimulation could affect the synaptic secretome. Interestingly, it has been reported that treatment with LP-211 selectively increases the secretion of cystatin B from synaptosomes, but does not affect the secretion of other proteins, such as Tau, an axonal protein whose neuronal and synaptic secretion has recently been demonstrated (Dubai 2018; Brunello et al 2018; Pernègre et al. 2018), and CD81, a marker of exosomes, which are vesicles released by neurons into the extracellular space (Janas et al 2016) (Figure 12 A-D). In addition, the secretion in the medium of β-actin, a cytoskeletal protein often found associated with exosomes, was not affected by LP-211 (Figure 12 A-E). Altogether, these results indicate that 5-HT7R activation selectively modulates the secretion of specific proteins, suggesting a fine tuning of synaptic plasticity exerted by the signaling pathway associated with this receptor.

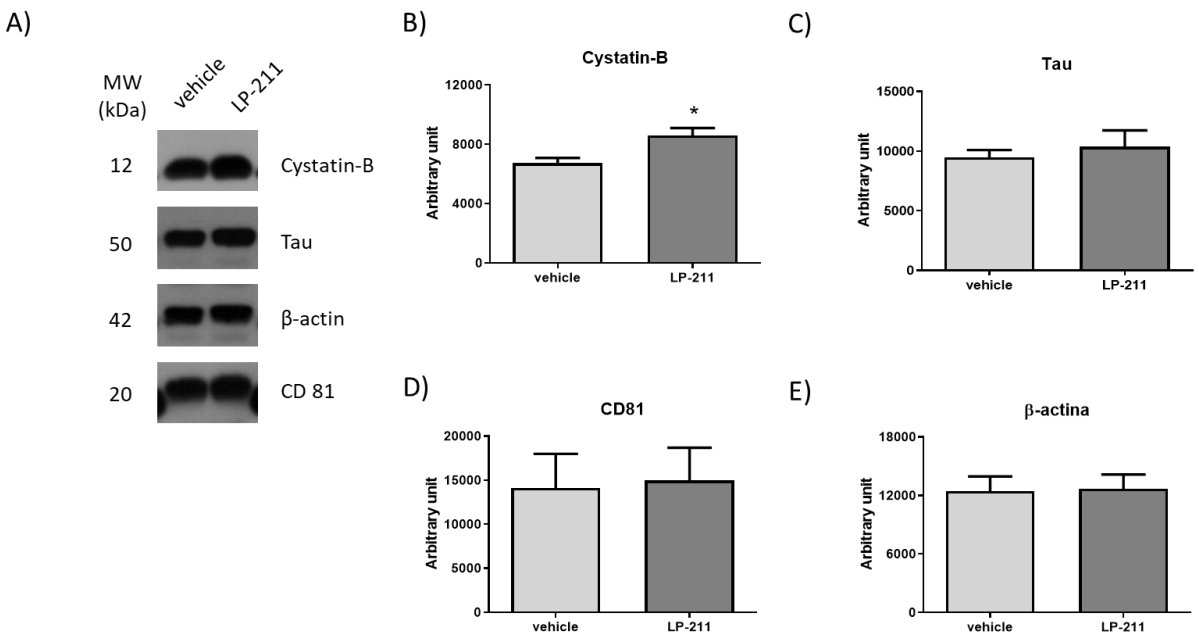



**Figure 12: Effect of LP-211 on extracellular levels of proteins secreted by the synaptosomes**. A) Representative images of the Western Blot analysis of secretome from synaptosomes incubated with or without LP-221 using antibodies against cystatin B, Tau, CD81 and β-actin. Expression levels of B) cystatin-B, C) Tau, D) CD81 and E) β-actin, in the secretome of synaptosomes incubated for 2 hrs with 100 nM LP-211 or vehicle (DMSO). Data are means ± SEM of n=4. T-test for paired data. *p-value <0.05. MW: molecular weight; kDa: kDalton.

### 5.4 5-HT7R in human cerebral organoids: preliminary data

To extend our study to human models, we took advantage of the 3D human multicellular model of brain cortical tissue, namely cerebral organoids (Lancaster et al., 2013; Lancaster and Knoblich, 2014; Camp et al., 2015). We prepared crude synaptosomal fraction (P2) from two and five months old human brain organoids (hCO; pool of 20–40 organoids), and we compared the expression level

of synaptophysin, a typical presynaptic protein, in the P2 fraction compared to the homogenate (Figure 13). Interestingly, synaptophysin was highly enriched in the crude synaptosomal fraction, indicating that this fraction is a good model system to study *in vitro* the synaptic region of hCOs. On the other hand, β-actin was equally distributed in the homogenate and synaptosomal fractions of hCO, as expected for a ubiquitous cytoskeletal protein (Figure 13). These data are comparable to the ones obtained from rodent brains fractions (Penna et al 2019). Interestingly, 5-HT7R is present in hCO homogenate as well as in P2 fraction, and its expression level seems to decrease during developmental stages from 2 to 5 months. These data indicate, for the first time, the presence of 5-HT7R in human brain organoids and open the way to new studies aiming at investigating the role of 5-HT7R in neuronal plasticity in a human brain model.

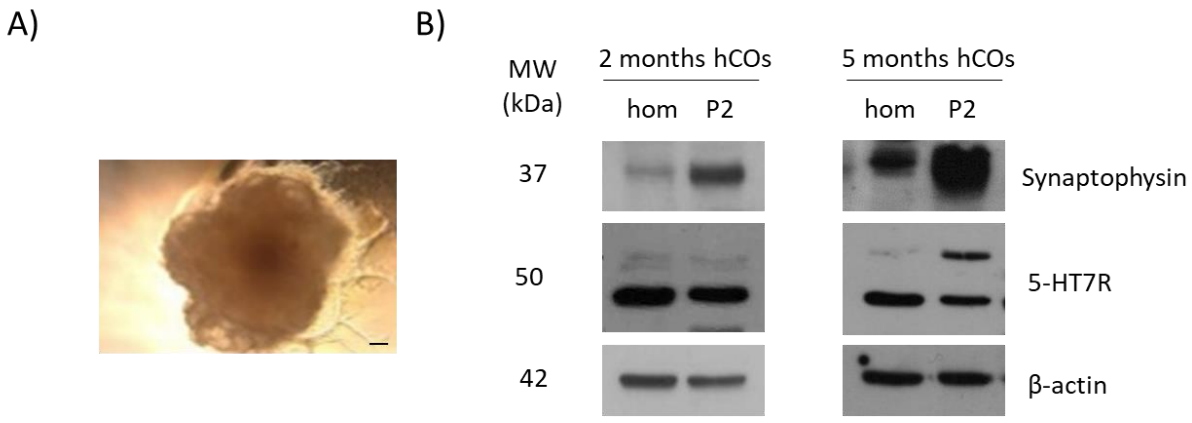



**Figure 13**: **Presence of 5-HT7R in human cerebral organoids.** A) Representative images of the hCOs (from Di Matteo et al. 2020). B) Relative intensity of 5-HT7R, synaptophysin and β-actin from two and five months old hCOs

## 5.5 Effects of 5-HT7R activation on electrophysiological and behavioural impairment in the Angelman Syndrome animal model

### *5.5.1 LP-211 reverses LTP impairment in field CA1 of hippocampal slices from AS mice*

Previous findings have shown that AS mice show impairment in TBS-induced LTP in field CA1 of hippocampal slices (Baudry et al., 2012; Liu et al., 2019). Indeed, we confirmed that in AS mice TBS produced only short-term potentiation, with fEPSP slope returning to baseline levels in about 30 min (Figure 14 A-B). We tested the effects of LP-211 on TBS-induced LTP in field CA1 of acute hippocampal slices from WT and AS mice (Figure 14 A, B). Interestingly, incubation of slices with LP-211 (1 μM) for 30 min resulted in recovery of TBS-induced LTP in AS mice. LP-211 treatment did not modify TBS-induced LTP in hippocampal slices from WT mice. We also verified that LP-211 (1 μM) did not affect baseline recording in both WT and AS mice (Figure 15 A, B).

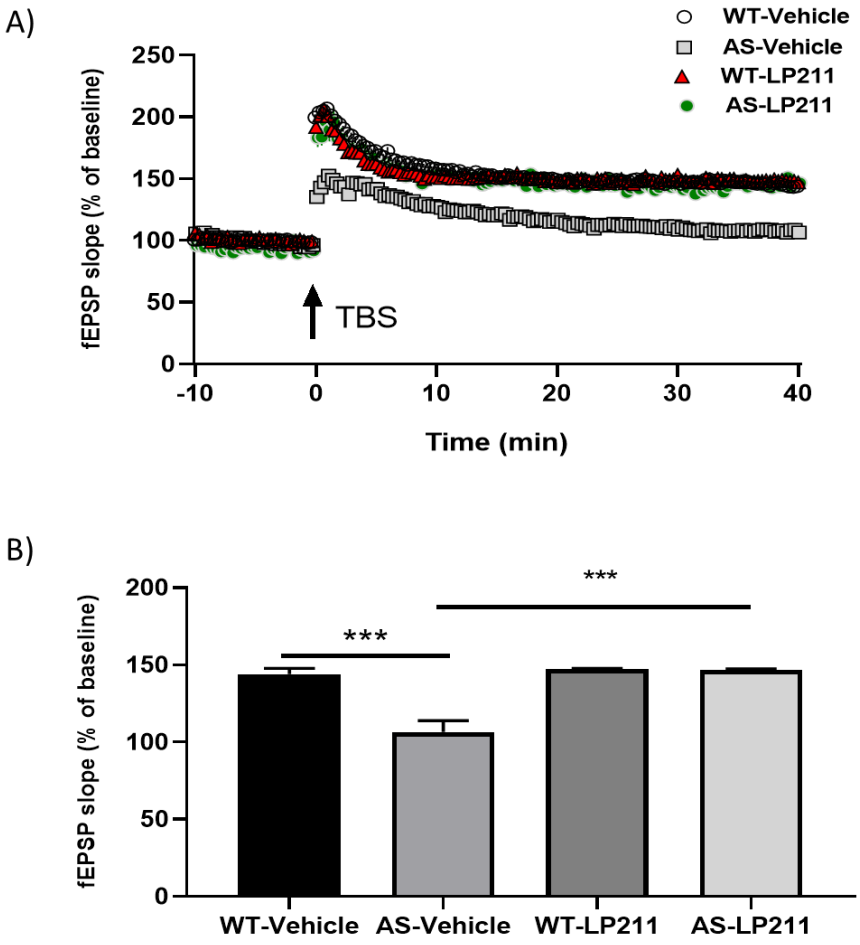



**Figure 14**. **Effects of LP-211 on TBS-induced LTP in CA1 field of hippocampal slices from wild type (WT) and Angelman syndrome (AS) mice**. A) Acute slices from the hippocampus of WT and AS mice were treated with vehicle or LP-211 (1µM) 30 minutes before TBS. fEPSP slopes were calculated as percent of fEPSP slopes averaged over the 10 min baseline period. LTP amplitude was measured 40 min after TBS as the percent of the fEPSP slopes averaged over 10 min baseline period. B) Results are presented as means ± SEM of values obtained in 6 slices from 3-5 mice, *** $p<0.001$ as compared to WT-Vehicle, ###$p<0.01$ as compared to AS-Vehicle group.

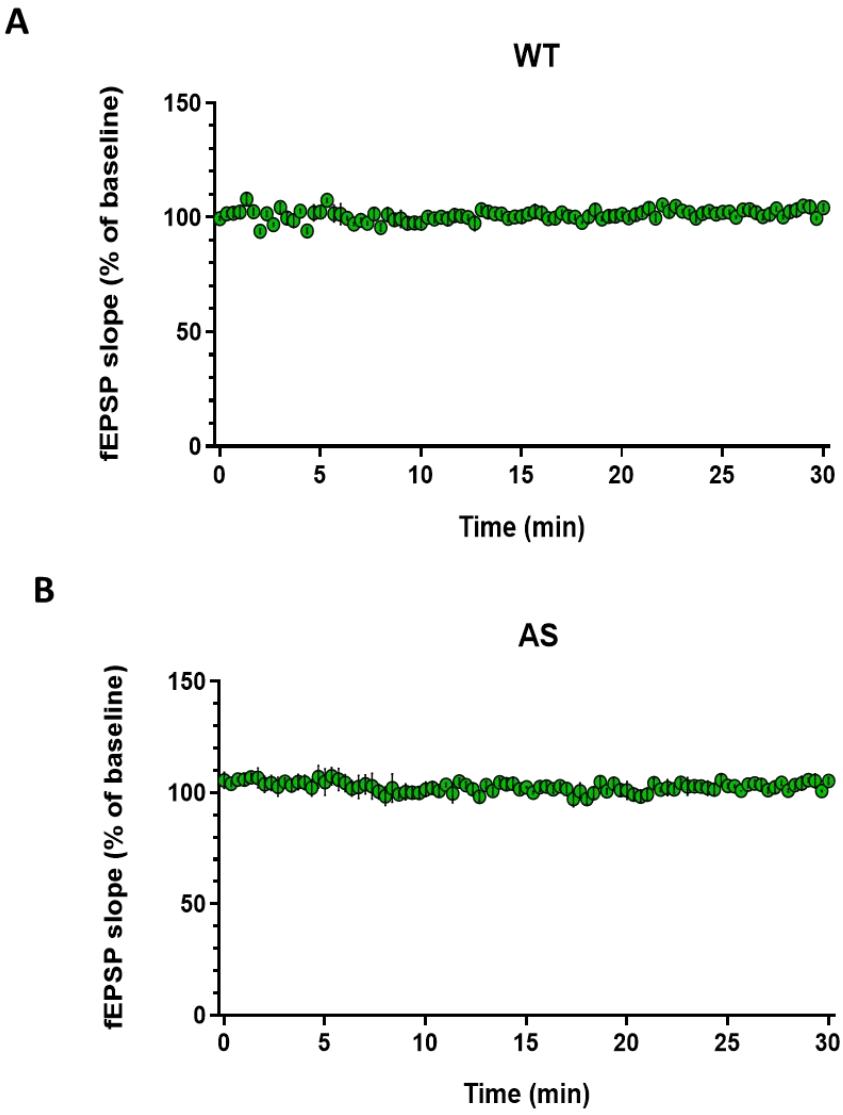



**Figure 15**. **Effect of LP-211 on baseline recording in CA1 field of hippocampal slices from wild type (WT) and Angelman syndrome (AS) mice.** fEPSP slopes were calculated as percent of fEPSP slopes averaged over the 10 min baseline period before adding LP-211 (1µM). A) Baseline recording in CA1 field of hippocampal slices from WT mice. B) Baseline recording in CA1 field of hippocampal slices from AS mice. N=3 for each group.

### *5.5.2 LP-211 treatment reverses cognitive deficits in AS mice.*

It has been previously reported that AS mice, trained for both the context and tone test of the fear-conditioning paradigm, exhibit learning and memory impairment (Liu et al 2019, Baudry et al 2012, Sun et al 2016). We confirmed these results (Figure 16 A, B) and tested the capacity of LP-211 to reverse the impairment. Male WT and AS mice were injected with vehicle or LP-211 (3 mg/kg) 30 minutes before training in fear-conditioning. Interestingly, in the context version of the test, AS mice treated with LP-211 exhibited a significant increase in freezing time, as compared to AS mice treated with vehicle, although their response was still lower than the one in LP-211-treated WT mice. LP-211 treatment did not affect freezing response of WT mice (Figure 16 A). On the other hand, when male animals were tested in the tone version of the test, the impaired performance of AS mice was not reversed by LP-211 treatment (Figure 16 B). No difference in freezing time was detected in the pre-conditioning period, or before the tone application in all experimental groups.

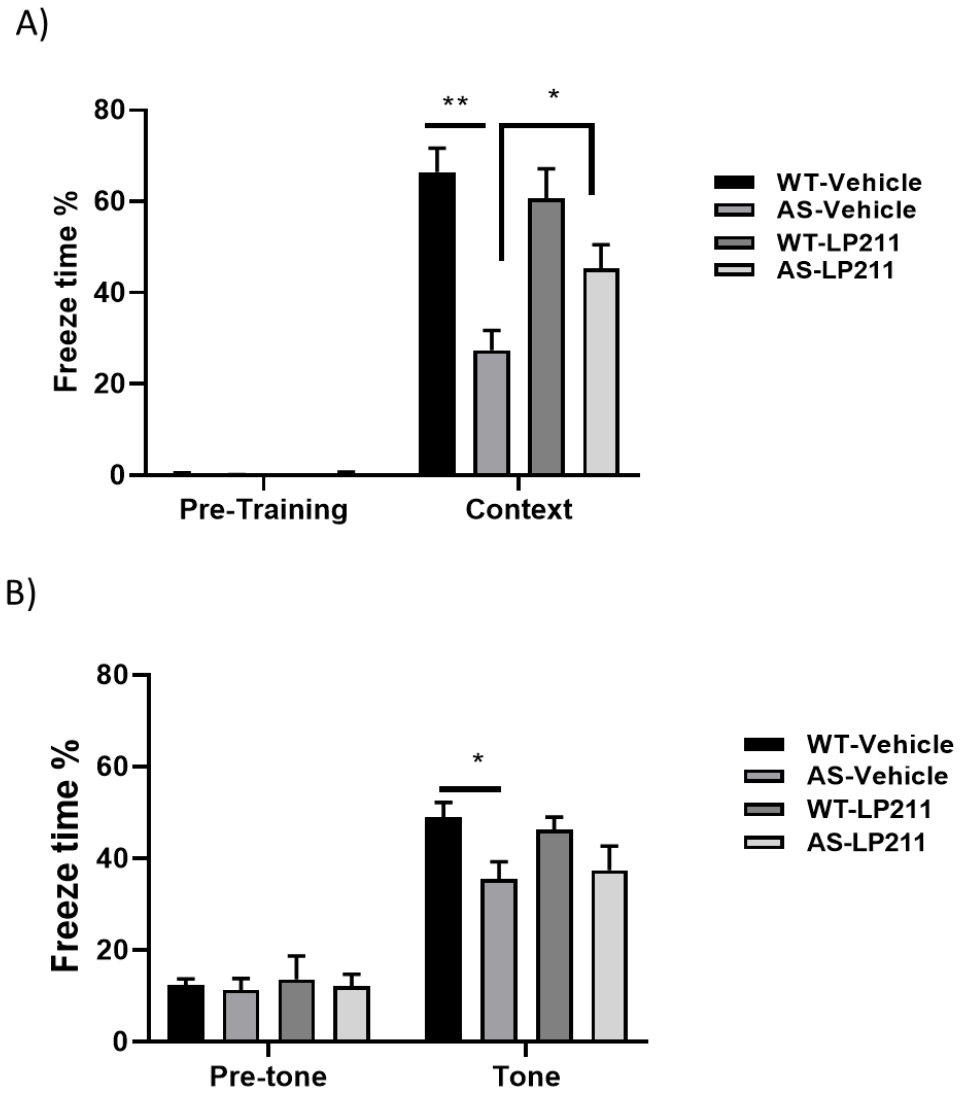



**Figure 16. LP-211 reverses impairments in contextual fear conditioning of AS male mice**. WT and AS male mice were treated with vehicle or LP-211 (3 mg/kg, ip) 30 minutes before being trained for fear conditioning. A) % freezing time for different experimental groups in context memory. B) % freezing time for different experimental groups in tone memory. Data are means ± SEM of n=4 mice; * $p<0.05$ as compared to AS-Vehicle,** $p<0.001$ as compared to AS-Vehicle .

Similar results were obtained when female animals were tested with the same experimental paradigm (Figure 17). Interestingly, at variance with male animals, LP-211-AS treated females exhibited the same performance than WT animals in the context version of the test. Thus, LP-211 was able to completely reverse the learning impairment of AS female mice. On the other hand, in female mice, as in males, LP-211 treatment did not have any effect on the impaired performance of the animals in the tone version of the test (Figure 17 A and B).

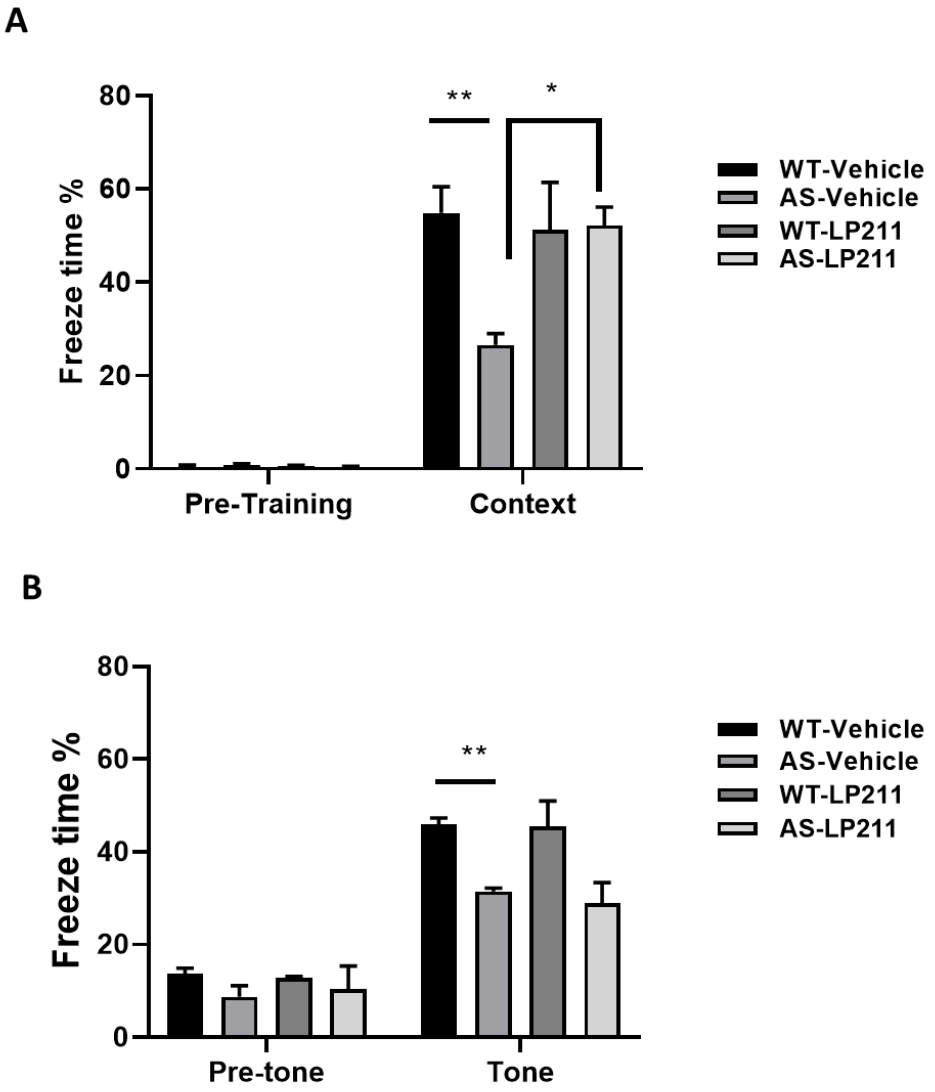



**Figure 17 LP-211 reverses impairments in contextual fear conditioning of AS female mice**. WT and AS female mice were treated with vehicle or LP-211 (3 mg/kg, ip) 30 minutes before being trained for fear conditioning. A) % freezing time for different experimental groups in context memory. B) % freezing time for different experimental groups in tone memory. Data are means ± SEM of n=3 for WT-Vehicle group and n=4 for other groups. *p <0.005, as compared to AS-Vehicle; **p <0.001, as compared to AS-Vehicle mice.

## 6. SUMMARY OF RESULTS

In conclusion, the experimental results obtained during my PhD show that:

1) The binding kinetic of arylpiperazine-based 5-HT7R ligands correlates better to the lipophilicity of the aryl moiety linked to the piperazine ring than to the lipophilicity of the terminal fragment linked to the alkyl chain. Moreover, it was also demonstrated that the position of polar groups on the aryl moiety linked to the piperazine ring affects the residence time.
2) The residence time of 5-HT7R ligands correlates with their biological activity, at least with regards to neurite elongation.
3) The stimulation of 5-HT7R with LP-211 in a synaptosomal fraction from brain cortex finely regulates processes involved in synaptic plasticity, such as local synthesis and secretion of selected proteins.
4) 5-HT7R is present in the synaptic fraction of human cerebral organoids at very early stages of development.
5) The stimulation of 5-HT7R with LP-211 rescues LTP and behavioral impairment in an animal model for Angelman syndrome

All together, these data broaden the knowledge on the molecular mechanisms linked to 5-HT7R activation and pave the way for the development of new 5-HT7R ligands, which may be of potential interest for pharmacological treatment of neurodevelopmental diseases characterized by altered connectivity as Angelman syndrome, and in general Autism Spectrum Disorders.

## 7. DISCUSSION AND CONCLUSIONS

A wealth of data has demonstrated that 5-HT7R activation modulates neuronal morphology, excitability, and plasticity, thus contributing to shape brain networks during development and to remodel neuronal connectivity in fully developed adult brain (Crispino et al 2020). Therefore, 5-HT7R activation has been proposed as a valid therapeutic approach for neurodevelopmental and neuropsychiatric disorders associated with abnormal synaptic plasticity and neuronal connectivity (Crispino et al 2020). The findings described above strengthen and extend previous data, strongly supporting this hypothesis.

Considering the potential therapeutic role of 5-HT7R ligands and the increasing importance of drug-receptor binding kinetic parameters for the evaluation of the biological efficacy of drugs *in vivo* and for the development of next-generation drugs, we investigated the binding kinetics of a set of 5-HT7R ligands. It is noteworthy that selecting drug candidates based on equilibrium-derived parameters alone, such as affinity (Ki), may be problematic since the dynamic events occurring in *vivo*—absorption, distribution, metabolism, and excretion—often prevent the free drug from reaching equilibrium conditions (Guo et al 2014).

We described structure-kinetics relationships of a set of arylpiperazine-based 5-HT7R ligands. We found that the lipophilicity of the aryl moiety linked to the piperazine ring had a greater impact than the lipophilicity of the terminal fragment linked to the alkyl chain on the kinetics of ligands binding interaction with 5-HT7R. In addition, the position of polar groups on the aryl moiety linked to the piperazine ring had an impact on the residence time. These data provided, for the first time, information on the effects of the physicochemical properties of specific substituents on binding kinetics of 5-HT7R ligands with an aryl piperazine structure.

To verify the biological effects of 5-HT7R ligands with different residence times, we studied their impacts on the neurite elongation in primary neuronal cultures. The results were compared to the ones obtained with LP-211, which was previously demonstrated to promote neurite outgrowth in neuronal primary cultures (Speranza et al 2013, 2015, Volpicelli et al 19). We found that the 5-HT7R agonist **4**, with a $K_i$ value very close to that of LP-211 and residence time about one third of LP-211 (8.7 min versus 24 min for LP-211), induced neurite elongation in neuronal primary cultures with a different timing, as compared to LP-211. In particular, its effect started earlier and terminated more rapidly than that of LP-211. These results suggest that the residence time of a ligand positively correlates with its biological activity, at least with regards to the effect on neurite elongation. On the other hand, compound **6**, with a higher residence time (33 min), did not exhibit a significant effect on neurite outgrowth with 4 h incubation. It is possible to hypothesize that compound **6**, due to its longer residence time, requires a longer incubation time to display its effect on the morphology of neurites. On the other hand, it cannot be ruled out that compound 6 is a 5-HT7R antagonist, in spite the very high structural resemblance to compound **4**. In addition, it is worth to consider that compound **6** has a lower affinity ($K_i$= 57 nM) than LP-211 ($K_i$= 20 nM) and compound **4** (Ki= 19.3 nM). These data reported here represent the first attempt to shed light on the relationship between residence time and pharmacological activity, in relation to a potential therapeutical treatment. Further studies are yet required.

To further investigate the effects of 5-HT7R stimulation on synaptic plasticity, we incubated synaptosomes isolated from rat cerebral cortex with LP-211. Interestingly, we found that LP-211 stimulation of 5-HT7R activated the ERK pathway in synaptosomes, in agreement with data showing LP-211-dependent ERK activation in neuronal primary cultures (Speranza et al 2013).

In contrast, 5-HT7R stimulation did not lead to the activation of mTORC1 and mTORC2 pathways in synaptosomes, since no difference in the phosphorylation of their downstream effectors (P70 and AKT, respectively) was detected following incubation with LP-211. Nonetheless, the phosphorylation of P70 increased significantly when synaptosomes were incubated for 2 h in presence or absence of LP-211, as compared to non-incubated fractions. Synaptosomes represent an *in vitro* model of synaptic regions and have been extensively used by our research group to study local protein synthesis (Eyman et al 2007, 2013, Cefaliello et al 2019, Perrone-Capano et al. 2021). Interestingly, the mTORC1 pathway is well-known to promote protein synthesis (Laplante and Sabatini, 2012). Thus, P70 phosphorylation in incubated synaptosomes may reflect the activation of local protein synthesis in synaptosomes.

To verify the possible effect of 5-HT7R activation on synaptic protein synthesis, we analysed expression levels of eIF2B2, an initiation factor of eukaryotic protein synthesis, in synaptosomes incubated with LP-211 or with vehicle. eIF2B2 is locally synthesized in axons, and its axonal downregulation inhibits the translation of specific axonally localized mRNAs (Kar et al., 2013). Surprisingly, eIF2B2 expression decreased dramatically following treatment with LP-211, suggesting that stimulation of 5-HT7R inhibits local translation. In a similar way, expression of cystatin-B, a protein locally synthetized in the synaptosomal fraction (Penna et al 2019), decreased significantly in synaptosomes treated with LP-211, suggesting that also its local synthesis was inhibited. On the other hand, expression of cytoskeletal proteins such as β-actin and α-tubulin, did not change following incubation with LP-211, indicating that activation of 5-HT7R receptor specifically influences the local synthesis of specific proteins rather than having a generalized inhibitory effect on local protein synthesis.

Since synaptic plasticity also depends on the secretion of proteins through vesicular release, we analysed possible changes in the synaptic secretome following stimulation of 5-HT7R. Our results indicated a selective increase of cystatin-B secretion from synaptosomes incubated with LP-211, while the secretion of other proteins was not affected. In conclusion, in the presence of LP-211, the expression level of cystatin-B in synaptosomes decreased, while its secretion increased, suggesting a role of 5-HT7R in the synaptic turnover of this protein. A recent elegant study confirmed the involvement of 5-HT7R in synaptic plasticity, demonstrating the role of 5-HT7R signaling in presynaptic modulation. In particular, it was shown that 5-HT7R activation drastically reduced the number of synaptic vesicles in human presynaptic nerve terminals and modulated neurotransmitter release (Patzke et al 2019).

Interestingly, we also detected the presence of 5-HT7R in synaptic fractions from hCOs prepared at different maturation stages. The receptor was already present at early stages and its expression changed during maturation, suggesting its involvement during brain development in human neurons. Accordingly, a very recent study indicated that the constitutive activity of another serotonin receptor, the 5-HT6R, was essential to maintain human neural stem cell multipotency, and regulated hCOs formation (Wang et al 2021). Considering that hCOs were used to study the pathogenesis of many diseases in which 5-HT7R is involved, such as Rett syndrome and Angelman syndromes (Hirose et al 2020; Nieto-Estevez and Hsieh 2020), our results provide further support to investigate the molecular mechanisms linked to the activation of the receptor, which could have a potential value as a diagnostic and identification of therapeutical target for these diseases.

In this perspective, we studied the effects of 5-HT7R activation in an animal model of Angelman syndrome. In particular, we focused on the hippocampus, an important limbic structure receiving

serotonergic inputs from raphe nuclei. 5-HT7Rs are present in both CA3 and CA1 region of the hippocampus, an area with a crucial role in learning and memory (Bonaventure et al 2002). We found that 5-HT7R activation by LP-211 was able to reverse the LTP impairment in hippocampal slices from AS mice. These results underscore the key role of 5-HT7R in modulating long term plasticity in hippocampus, in agreement with previous results indicating impaired LTP in hippocampal CA1 region of 5-HT7R knock out mice (Roberts et al 2004). Since LTP is currently accepted as a crucial mechanism underlying learning and memory, our next step was to investigate the effects of 5-HT7R stimulation on learning and memory in AS mice. These animals display a deficit in long-term memory when compared to WT, as indicated by the reduction in freezing scores to the context and to the tone, respectively of 50% and 30% (Baudry et al., 2012; Sun et al 2015a, 2016). Treatment with LP-211 selectively rescued the behavioural impairment in contextual fear conditioning but did not have a significant effect in tone fear conditioning. It is worth noticing that the rescue efficiency appeared to be gender-dependent, since LP-211 treated AS female mice performed better than male mice in the contextual fear conditioning task. We performed both contextual and tone fear conditioning to evaluate hippocampal-dependent and -independent learning (Kim and Fanselow, 1992). Indeed, the contextual fear conditioning depends on a neural circuit including the hippocampus, basolateral amygdala and medial prefrontal cortex (Maren et al 13), while cued fear conditioning appears to be hippocampus-independent (Phillips and LeDoux 1994), even if this issue is still matter of debate (Pignataro et al 2013). Although 5-HT7R are similarly distributed in different brain regions, including the amygdala and the hippocampus (Gustafson et al 1996; Neumaier et al 2004; Varnäs et al 2004), the data related to the effect of its stimulation on fear conditioning responses are somehow controversial. Indeed,

using 5-HT7R knock out mice, 5-HT7R were shown to be involved in contextual but not cued fear conditioning (Roberts et al 2004), in agreement with our data. On the other hand, administration of a 5-HT7R selective antagonist was shown to cause a significant decrease in both context- and tone-dependent fear conditioning (Matsushita et al 2009). These data confirmed the role played by 5-HT7R signaling in modulating behavioural responses, although further investigations are necessary to unveil the underlying molecular mechanisms in different brain regions.

Altogether, the results of my research project strengthened the involvement of 5-HT7R in plasticity of the nervous system and indicated that LP-211 is a very promising molecule to rescue synaptic plasticity and cognitive functions in AS. In general, these data provide new insights into the selection of innovative pharmacological compounds targeting this receptor for the treatment of neurodevelopmental disorders characterized by altered brain connectivity.

## 8. LIST OF ABBREVIATIONS

***A***

| | |
|---|---|
| AS | Angelman sindrome |
| ASD | Autism Spectrum Disorders |
| *ATP* | *Adenosine triphosphate* |

***B***

| | |
|---|---|
| BDNF | Brain-derived neurotrophic factor |
| *BSA* | *Bovine serum albumine* |

***C***

| | |
|---|---|
| cAMP | 3'-5'-cyclic adenosine monophosphate |
| clogD | calculated logarithm of partition coefficient |
| Cdk5 | Cyclin-dependent kinase 5 |
| Cdc42 | Cell division control protein 42 homolog |
| CHX | Cycloheximide |
| CNS | Central Nervous System |
| CTRL | Control |
| CTX | Cortex |

***D***

| | |
|---|---|
| DAPI | 4',6-diamidino-2-phenylindole |
| DIV | Days *in vitro* |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |

***E***

| | |
|---|---|
| eIF2B2 | Eukaryotic protein synthesis |
| E | Embryonic age |
| ERK | Extracellular signal-regulated kinase |
| EPSP | Excitatory postsynaptic potential |

***F***

| | |
|---|---|
| FBS | Fetal bovine serum |
| FXS | Fragile X syndrome |
| fEPSP | Field excitatory postsynaptic potentials |

***G***

| | |
|---|---|
| G418 | Geneticin |
| GABA | Gamma-aminobutyric acid |
| GPCR | G-protein coupled receptor |

| | |
|---|---|
| Gs | Activating adenylyl cyclase |
| *GTP* | *Guanosine-5'-triphosphate* |

***H***

| | |
|---|---|
| hCO | Human cerebral organoid |
| HIPP | Hippocampus |
| HM | Homogenization medium |

***I***

| | |
|---|---|
| IC50 | Half maximal inhibitory concentration |

***K***

| | |
|---|---|
| *Ki* | *Constant of inhibition* |
| KO | Knockout |
| Kob | Observed association rate constant |
| koff | Constant dissociation rate |
| kon | Constant association rate |

***L***

| | |
|---|---|
| LP-211 | N-(4-cyanophenylmethyl)-4-(2-diphenyl)-1-piperazinehexanamide |
| LTD | Long-term depression |

| | |
|---|---|
| LTP | Long-term potentiation |

***M***

| | |
|---|---|
| mGluR | Metabotropic glutamate receptors |
| mRNA | Messenger ribonucleic acid |
| mTOR | Mechanistic target of rapamycin |
| mTORC | Mechanistic target of rapamycin complex |

***P***

| | |
|---|---|
| p-ERK | Phosphorylated-Extracellular signal-regulated kinases |
| P | Postnatal day |
| P2 | Crude synaptosomal fraction |
| PAGE | Polyacrylamide gel electrophoresis |
| *PBS* | *Phosphate buffered saline* |
| PKA | Protein kinase A |
| PSD-95 | Postsynaptic density protein-95 |
| PVDF | Polyvinylidene fluoride |

***R***

| | |
|---|---|
| RNA | Ribonucleic acid |
| RT | Residence time |

| | |
|---|---|
| RTT | Rett syndrome |

***S***

| | |
|---|---|
| SB-269970 | (2R)-1-[3-hydroxy-phenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidine |
| SDS | Sodium dodecyl sulfate |
| SEM | Standard error of mean |
| SERT | Serotonin reuptake transporter |
| SK2 | Calcium-activated potassium channels |
| SSRIs | Sselective serotonin reuptake inhibitors |
| STR | Striatum |
| SYN | Synaptosomes |

***T***

| | |
|---|---|
| Tph | Tryptophan 5-hydroxylase |
| TBS | Tris-buffered saline |
| TBS | Theta burst stimulation |
| TBST | Tris buffered saline + Tween |

***U***

| | |
|---|---|
| UBE3A | Ubiquitin-protein ligase E3A |

***V***

| | |
|---|---|
| V | Volt |

***W***

| | |
|---|---|
| WB | Western blot |
| WT | Wild type |

| | |
|---|---|
| 5-CT | 5-carboxamidotryptamine |
| 5-HT | 5-hydroxytryptamine |
| 5-HTP | 5-hydroxy-L-tryptophan |
| 5-HTR | 5-HT receptor |
| 5-HT7R | 5-HT receptor 7 |
| 8-OH-DPAT | 8-hydroxy-N,N-dipropylaminotetraline |

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