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Altered synaptic plasticity in neurodevelopmental and neurodegenerative diseases

PHD STUDENT: AMELIA PIZZELLA

TUTOR: PROF.SSA MARIANNA CRISPINO

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1. ABSTRACT

Synaptic plasticity refers to activity-dependent adjustments of neuronal circuits which enable remodeling of preexisting synaptic connections to change strength or efficiency of synaptic transmission and thereby modulate neuronal excitability. The growing evidence showing the ability of nerve endings to synthesize proteins independently from the cell body provides a new perspective for synaptic plasticity. Indeed, synapses can respond to the local stimuli by changing quickly and appropriately their proteins pattern. Moreover, recent studies suggested that synaptic plasticity may also be mediated by the release of extracellular vesicles from synaptic terminals. In addition to regulating the extracellular neuronal environment, these extracellular vesicles mediate long distance cell-cell communication. Interestingly, altered mechanisms of synaptic plasticity has been demonstrated in various neurodegenerative and neurodevelopmental (NDDs) pathologies, associated with synaptic dysfunctions and therefore referred to as synaptopathies. This thesis aims to unveil potential common mechanisms underlying these neuropathologies, and to identify novel therapeutic target. As an *in vitro* model of nerve endings detached from neuronal soma, synaptosomal fraction was isolated from the cerebral cortex of two different animal models for NDDs, i.e. BTBR mouse for Autism Spectrum disorder (ASD), and Ube3A$^{tm1Alb/J}$ mouse for Angelman syndrome (AS). The results of biochemical and morphological analyses indicated that the synaptic system of protein synthesis was deregulated in both NDDs. This investigation was extended to a neurodegenerative disease that displays neuronal impairments already during neurodevelopment, the Myoclonic Epilepsy type 1 (EPM1). In particular, synaptosomal fraction was isolated from human cerebral organoids

(hCOs) generated from induced pluripotent stem cells (iPSCs) of EPM1 patients. In this human model of synaptic regions, it was demonstrated a deficit of the synaptic system of protein synthesis, but also an alteration of extracellular vesicles release from nerve endings.

A potential candidate for the treatment of synaptopathies is the receptor 7 of serotonin (5-HT7R), which is tightly involved in neuroplasticity. Indeed, in this study we demonstrated that stimulation of 5-HT7R with a selective agonist was able to reverse the synaptic deficits in ASD and AS.

Altogether, this work indicates the alterations of synaptic plasticity mechanisms as a common deficit in neurodevelopmental and neurodegenerative diseases for which targeting 5-HT7R could be an appropriate candidate for therapeutic strategy.

2. INTRODUCTION

2.1 Synaptic plasticity

To transfer information, neurons create complex and well-organized neural networks by use of synapses, the functional connection points between neurons. The most fascinating and important property of the mammalian brain is its ability to adapt itself in response to external stimuli and injuries through morphological and functional modifications of neuronal connections (Fernandes & Carvalho, 2016). This property called 'synaptic plasticity' involves adjustments of neuronal circuits, including variations in the number of synaptic contacts, remodelling of preexisting neuron-to-neuron connections with changes in the strength or efficacy of synaptic transmission, and subsequent modulation of neuronal excitability. The phenomenon of synaptic plasticity manifests not only during development but also throughout adulthood (Citri & Malenka, 2008). Some crucial cognitive functions, such as learning and memory, depend on activity-dependent modifications in synaptic efficacy, as long-term depression (LTD) and long-term potentiation (LTP) (Malenka & Bear, 2004; Rioult-Pedotti et al., 2000). Synaptic remodeling occurs through rearrangements of neuronal morphology at both presynaptic and postsynaptic levels. This dynamic reorganization is crucial for the development of cognitive strategies, for the functional recovery processes of brain areas following damage, and for slowing down the age-associated neurodegenerative processes (Fridman, 2004; Heuninckx et al., 2008). Numerous molecular and cellular mechanisms have been demonstrated to be involved in synaptic plasticity. Interestingly, the protein synthesis system located in the synaptic areas has been shown to participate in this process (Crispino et al., 2014; Giuditta et al., 2008; Holt et al., 2019; Perrone-Capano

et al., 2021). Moreover, recent data underscore that extracellular vesicles (EVs) secreted by synaptic terminals for long distance cell-cell communication are also important contributors to synaptic plasticity (Di Giaimo et al., 2020).

2.1.1 Presynaptic protein synthesis

Synaptic plasticity refers not only to the physical rearrangement of subcellular compartments such as dendrites, axons, and nerve terminals but also to the modulation of the strength of synaptic connections, which requires rapid and regulated modulation of the local proteome. In this regard, the protein synthesis apparatus located in axonal and presynaptic areas that is activated on-site and on-demand has a crucial role in remodeling synaptic regions. This feature makes it a perfect candidate for a molecular mechanism contributing to synaptic plasticity (Giuditta et al., 2008; Holt et al., 2019; Perrone-Capano et al., 2021).

The idea that protein synthesis takes place not only in neuronal cell bodies, but also in axonal and presynaptic territories took a long time to be accepted, after it was initially proposed in the 1960s (Giuditta et al., 2008; Perrone-Capano et al., 2021). There is now a consensus that axons and presynaptic endings do have the capacity for local translation of mRNA, which is more active during development but is retained into adulthood (Biever et al., 2019; Holt et al., 2019; Jung et al., 2014; Perrone-Capano et al., 2021; Shigeoka et al., 2016).

Local protein synthesis in presynaptic terminals has been often studied using the synaptosomal fraction of the brain (Ibrahim et al., 2023; Whittaker, 1993). Synaptosomes are produced by mild homogenization of brain tissues followed

by subcellular fractionation, and contain all the components corresponding to synaptic regions *in vivo* without the cell bodies (Whittaker, 1993). Using synaptosomes in combination with several other methodological approaches, it was demonstrated that synaptic translation plays a key role in controlling axonal shape, branching, and synaptogenesis during brain development (Holt et al., 2019; Perrone-Capano et al., 2021). Synaptic translation also controls the release of neurotransmitters during long-term plasticity in mature mammalian brains (Younts et al., 2016), plays a key role in remodeling synaptic regions of *Xenopus Laevis* embryos (Cagnetta et al., 2018), and is itself modulated by learning and memory in adult and old rats (Eyman et al., 2007, 2013).

Interestingly, a wealth of data demonstrated that deregulation of synaptic protein synthesis is involved in various neuropathologies characterized by impairment of synaptic functions, some of which are defined 'synaptopathies' (Castillo et al., 2023; C. J. Costa & Willis, 2018; Joo & Benavides, 2021; Lin & Yeh, 2020; Perrone-Capano et al., 2021; Sasaki, 2020).

2.1.2 Extracellular vesicles as cell-cell communication tools

Extracellular vesicles (EVs) are vesicles originating from the plasma membrane and secreted into the extracellular space. EVs are produced by most cells and play various roles in numerous physiological processes. In recent decades, EVs have emerged as a new tool for cell-cell communication (Raposo & Stoorvogel, 2013). Upon release, EVs can either act locally or travel in the extracellular space. EVs can interact with the extracellular matrix, and

eventually get internalized by target cells. The content of EVs can significantly diversify depending on cell type and developmental stage.

Most EVs are classified as exosomes or microvesicles based on their size, composition, and origin (Van Niel et al., 2018). Exosomes are small, homogeneous vesicles ranging from 40 to 100 nm in size, which originate from endocytic processes. Endocytic vesicles deriving from invagination of the plasma membrane, fuse to form early endosomes which mature into multivesicular bodies (MVBs). The MVBs contain intraluminal vesicles (ILVs), generated from inward budding of the MVBs membrane, and therefore have a cytoplasmic content. Instead of fusing with the lysosome, MVBs can fuse directly with the plasma membrane and release the exosomes into the extracellular space (Fig. 1). In contrast, microvesicles are produced directly through budding and fission from the plasma membrane and, therefore, their surface markers largely depend on the composition of the originating membrane. Additionally, they tend to constitute a broader and more heterogeneous population of extracellular vesicles, with a diameter ranging from 50 to 1000 nm.

EVs mediate intercellular communication in the central nervous system (CNS), where they are actively released by all cell types, including astrocytes (Verkhratsky et al., 2016), microglia (Paolicelli et al., 2019), oligodendrocytes (Frühbeis et al., 2013), neurons (Chivet et al., 2012) and, at the subcellular level, synapses (Di Giaimo et al., 2020; Olivero et al., 2021). In the brain, exosomes contribute to numerous processes including synaptic plasticity, response to neuronal stress, neurogenesis, neuroprotection and brain homeostasis (Saeedi et al., 2019; Schiera et al., 2019; Sharma et al., 2019; Smalheiser, 2007). Under physiological conditions, exosomes may transport

the information to establish correct neural connectivity and synaptic strength. For instance, synaptic AMPA and NMDA glutamatergic receptors, strongly involved in LTP, modulate the release of exosomes at synapses in differentiated neurons (Lachenal et al., 2011; Mittelbrunn et al., 2015). Furthermore, synaptic glutamatergic activity and calcium influx through NMDA and AMPA receptors regulate EVs production in neurons (Lachenal et al., 2011; Saeedi et al., 2019), suggesting that at least some signaling molecules linked to learning and memory may be secreted *via* EVs (Schiera et al., 2019).

The modification of EVs components mirrors the change in cell and tissues, and therefore their presence in biological fluids makes them a potential diagnostic marker of CNS pathology such as neurodegenerative diseases (Jin et al., 2021). Accordingly, several studies highlighted the significant implications of exosomal trafficking in the physiology and pathology of the CNS through the cellular spread of 'pathogenic proteins' as for instance β-amyloid peptides, tau, and prions (Caruso Bavisotto et al., 2019; Holm et al., 2018).

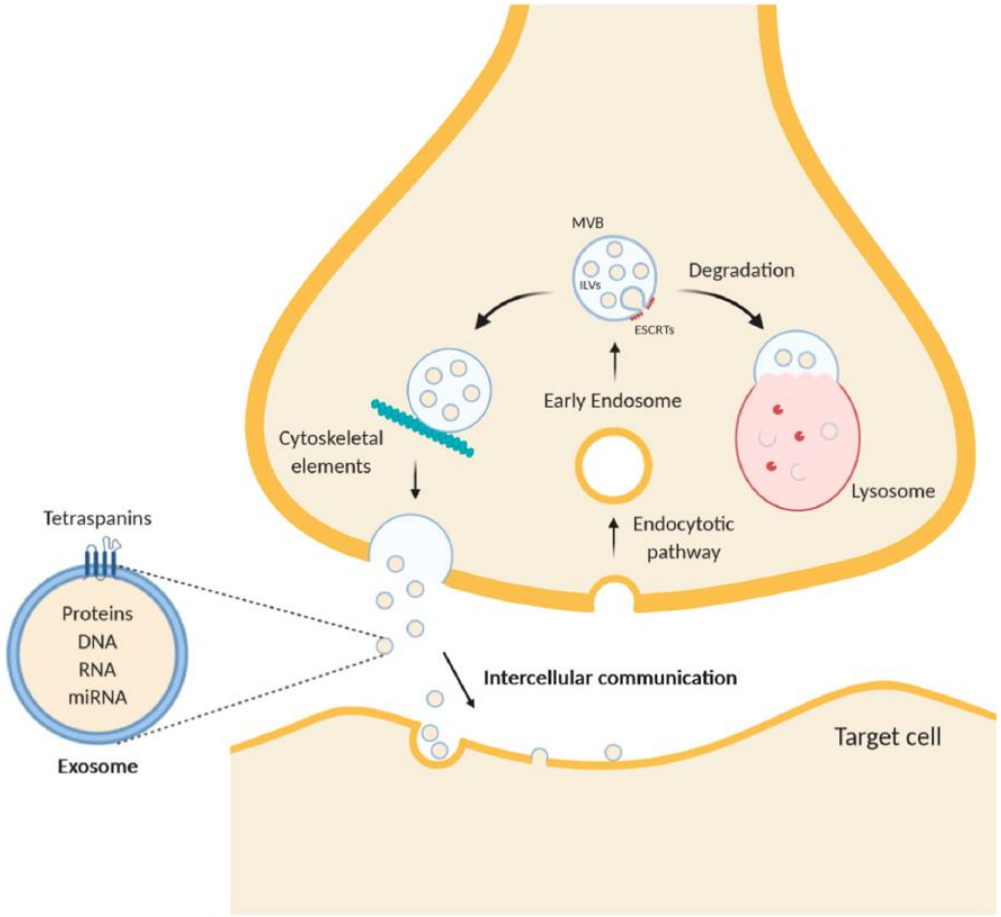


Figure 1: ***Schematic diagram of exosome trafficking in nerve endings (from Di Giaimo et al., 2020).*** As *multivesicular bodies (MVBs) progress through maturation, the inner membrane is remodelled to form the intraluminal vesicles (ILVs), which represent exosomes inside the MVBs. Following intracellular translocation facilitated by cytoskeleton elements like F-actin and microtubules, MVBs merge either with the plasma membrane, undergoing exocytosis, or with lysosomes for self-degradation. Exosomes contain in their*

lumen free proteins, RNA, microRNA, and DNA and are identified by transmembrane proteins belonging to the tetraspanins family.

2.2 The serotonin receptor 7 and synaptic plasticity

The serotonin receptor 7 (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, 5-HT7R is mainly expressed in the spinal cord, thalamus, hypothalamus, hippocampus, prefrontal cortex, striatal complex, amygdala, and in Purkinje neurons of the cerebellum (Crispino et al., 2020; Lippiello et al., 2016; Volpicelli et al., 2014). 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). 5-HT7R is a G protein-coupled receptor which activates at least two different signalling 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). cAMP activates protein kinase A (PKA), which 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 the stimulation of Rho GTPases, Cdc42 and RhoA. In turn, these intracellular signaling proteins, which 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 activation of Ca^{2+}/calmodulin pathways (Johnson-Farley et al., 2005; Lenglet et al., 2002).

Various lines of experimental evidence have highlighted the contribution of 5-HT7R in stabilizing synaptic connections during early embryonic and postnatal developmental phases (Crispino et al., 2020; Volpicelli et al., 2014). Additionally, activation of this receptor stimulates neurite outgrowth, thus modifying neural cytoarchitecture (Speranza et al., 2013). These effects are mediated by several signaling pathways, including mTOR, ERK, Cdc42, and Cdk5, which converge to modulate the expression and function of neuronal cytoskeletal proteins (Speranza et al., 2013, 2015; Volpicelli et al., 2014). Prolonged stimulation of the 5-HT7R pathway in adult mice and early postnatal mouse hippocampal neuron cultures has been shown to increase the number of dendritic spines and synaptic contacts (Speranza et al., 2013, 2017). Similar effects have been confirmed in brain circuits, specifically in organotypic slices prepared from young mouse hippocampi, where stimulation of the 5-HT7R pathway enhances dendritic spine formation, increases neuronal excitability, and modulates synaptic plasticity (Kobe et al., 2012). Stimulation of 5-HT7R in adolescent rats induces plastic rearrangements in brain circuits, potentially responsible for long-term behavioral changes in adulthood (Canese et al., 2015). Moreover, this receptor is implicated in cognitive functions such as learning and memory (Freret et al., 2014; Meneses, 2014; Roberts & Hedlund, 2012). Accordingly, dysfunctions of 5-HT7R have been demonstrated to be involved in numerous neuropsychiatric and neurodevelopmental diseases characterized by altered neuronal connectivity such as in Rett (RTT) and Fragile X syndrome (FXS) (L.

Costa et al., 2018; De Filippis et al., 2014, 2015). Collectively, these data clearly indicate the involvement of 5-HT7R in synaptic plasticity mechanisms.

2.3 Alteration of synaptic plasticity in neurodevelopmental disorders

Neurodevelopmental diseases (NDDs) are a heterogeneous group of childhood-onset diseases that impact the development of the nervous system, leading to abnormal brain functions. They include autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), intellectual disabilities, specific learning disorders, communication disorders and motor disorders (Mullin et al., 2013). In neurodevelopmental disorders, alterations of synaptic plasticity may trigger structural changes in neuronal circuits involved in cognitive functions (Sgritta et al., 2023). Dendrite and axon outgrowth is a critical step during development (Häusser et al., 2000) and alterations in dendritic structures have been noticed in several animal models of NDDs (Bourgeron, 2009; Cheng et al., 2017; M. Jiang et al., 2013; Martínez-Cerdeño, 2017), suggesting a close relationship between altered neuronal morphology and these diseases (Lee et al., 2021). Studies on animals have shown that many genes linked to increased risk of autism spectrum disorder are involved in neuronal activity, as their gene products modulate the strength and number of synapses (Ebert & Greenberg, 2013; Toro et al., 2010). Thus, mutations of these genes may lead to altered neuronal connectivity and plasticity (Hahamy et al., 2015), ultimately increasing the risk of ASD (Auerbach et al., 2011). Moreover, neurons lacking the fragile X mental retardation protein (FMRP), the main cause of FXS, display altered synaptic connectivity, suggesting that FMRP regulates the formation and/or

stabilization of neuronal connections (Bureau et al., 2008; Hanson & Madison, 2007). From the molecular point of view, it has been recently reported that disrupting the Brain Derived Neurotrophic Factor (BDNF)/ Tropomyosin receptor kinase B (TrkB, BDNF receptor) signaling pathway during a critical developmental period leads to NDDs (Sgritta et al., 2023).

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

2.3.1 Autism spectrum disorders and serotonergic signaling

Autism spectrum disorder (ASD, OMIM 209850) refers to a heterogeneous group of NDDs characterized by impaired social interaction and communication, repetitive and stereotyped behaviours, often accompanied by cognitive defects (Sztainberg & Zoghbi, 2016). Although many studies have been conducted since ASD was discovered, the aetiology and precise pathological mechanisms are still unknown. Current evidence indicates that the disorder's occurrence and progression are influenced by a combination of genetic and environmental factors (Bölte et al., 2019; Yoon et al., 2020). ASD is not associated with an alteration in a specific brain region, but rather results from an abnormal overall organization of the brain during development. Compared to normally developing children, those with ASD have accelerated brain development in the early stages of life, which leads to altered connectivity (Lewis et al., 2014). Specifically, mutations associated with ASD

influence the structure and turnover of synapses at various levels, as mutated genes encode proteins involved in chromatin remodeling and transcription, protein synthesis and degradation, actin cytoskeleton dynamics, and synaptic transmission (Bourgeron, 2015). Interestingly, it has been suggested that impaired synaptic protein synthesis may be one possible mechanism contributing to ASD (Kelleher & Bear, 2008).

Another pathway that has been widely demonstrated to be altered in ASD patients, as well as in various animal models of the disease, is the brain 5-HT neurotransmission system (Garbarino et al., 2019; Muller et al., 2016). In particular, the high serotonin levels detected in the plasma and platelets of ASD patients suggested the implication of serotonergic signaling in the pathophysiology of the disease (Cook & Leventhal, 1996; Schain & Freedman, 1961). Mice lacking 5-HT in the brain display typical phenotypes such as growth retardation and high aggressive behaviour, as well as other selective deficits, like impairment in social interactions and repetitive behaviour, which are features of ASD (Pratelli & Pasqualetti, 2019). Interestingly, it has been demonstrated that the ASD phenotype depends on variants in the gene encoding for 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). Moreover, changes in densities of serotonin receptors and transporters in different brain areas were detected in autistic patients (Brandenburg & Blatt, 2019; Oblak et al., 2013), and reduction of SERT binding capacity in the frontal cortex was found in autistic children (Makkonen et al., 2008). Different studies focused on evaluating therapeutic effects in ASD of selective serotonin reuptake inhibitors (SSRIs), which binds SERT and blocks 5-HT uptake, and are widely used for treating

psychiatric disorders (Kellner, 2010; Vaswani et al., 2003). However, so far there is no consensus regarding 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).

The 5-HT7R exhibits indirect connections with the disease, which make it a plausible target for treating ASD or improving associated symptoms and behavioral conditions (Ciranna & Catania, 2014). One study demonstrated that deep brain stimulation coupled with the administration of 8-OH DPAT, a 5-HT1AR/5-HT7R agonist, significantly alleviated hyperactivity and anxiety, and enhanced sociability in an animal model of ASD induced by valproate (VPA) (Wu et al., 2018). Consistently, other pharmacological treatments for ASD, like risperidone and lurasidone, which are used to mitigate aggressive behavior in ASD patients, act as antagonist of 5-HT7Rs (Loebel et al., 2016; Smith et al., 2011; Wishart, 2006). Although a wide range of antidepressant and antipsychotic drugs display high-affinity interactions with 5-HT7Rs (Leopoldo et al., 2011), none of the approved ASD drugs are highly selective for 5-HT7R, limiting its viability as a target for pharmacological treatment of ASD in humans. However, brain-permeant and selective agonists of 5-HT7R have effectively treated ASD dysfunctions in animal models of FXS and RTT. In particular, activation of 5-HT7R rescued synaptic plasticity, learning and autistic-like behaviour in adult FXS mice (L. 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 et al., 2014, 2015; Valenti et al., 2017). Therefore, 5-HT7R may be an interesting candidate

target to reverse some of the behavioural and molecular deficits associated with ASD.

2.3.2 Angelman syndrome: involvement of 5-HT7R

Angelman syndrome (AS, OMIM 105830) is a rare incurable neurodevelopmental disorder characterized by severe intellectual disability, impaired or absent speech, motor dysfunction, hyperactivity, autism-like behaviour, and seizure activities (Bi et al., 2016; Margolis et al., 2015; Tan & Bird, 2016). Other notable features include excessive inappropriate laughter and sleep disturbance (Bird, 2014). AS exhibits a high comorbidity with ASD, particularly in terms of developmental delay and language impairment (Hogart et al., 2010; Trillingsgaard & Østergaard, 2004). Developmental delay in AS individuals typically manifests within the first year of life, with language skills more delayed than motor skills. AS is characterized by absence of expression of the maternally inherited Ube3a gene located on chromosome 15q11.2 (Tan & Bird, 2016). Neurons rely entirely on the expression of Ube3a from the maternal allele, as the paternally inherited copy of the gene is silenced through genetic imprinting. The paternal Ube3a expression is suppressed by an atypical RNA polymerase II transcript (Bi et al., 2016; Meng et al., 2012). Due to the epigenetic imprinting, deletion or mutation of the Ube3a gene on the maternal chromosome results in the complete loss of the transcript.

The Ube3a gene encodes ubiquitin-protein ligase E3A, belonging to the family of E3 enzymes that regulate the process of protein ubiquitylation, one of the key posttranslational protein modifications (Rotin & Kumar, 2009).

Interestingly, it has been demonstrated that UBE3A functions not only as an E3 ligase in the ubiquitin-proteasome pathway (Greer et al., 2010), but also as a transcriptional co-activator for steroid hormone receptors (Nawaz et al., 1999; Ramamoorthy & Nawaz, 2008). However, it is not clear if both of these functions, when altered, are responsible for the phenotype of AS. Ube3a has been demonstrated to play a key role in neural circuit maturation and experience-dependent plasticity in the mammalian cerebral cortex (Sato & Stryker, 2010; Yashiro et al., 2009). A widely used animal model of AS is a mouse strain with maternal Ube3a deficiency, mirroring the AS features such as motor dysfunction, inducible seizures, and deficits in learning and memory (Y. Jiang et al., 1998; Van Woerden et al., 2007).

Despite the recognized comorbidity between AS and Autism Spectrum Disorder (ASD), and the established role of 5-HT in ASD, only a limited number of studies have directly explored the connection between 5-HT and AS. One of these studies revealed elevated 5-HT levels in the striatum and cortex of maternal Ube3a-deficient mouse compared to controls (Farook et al., 2012). In another study, chronic administration of the SSRI fluoxetine was shown to mitigate both molecular and behavioural deficits in AS mice (Godavarthi et al., 2014). These findings emphasize the involvement of the serotonergic system in the molecular and cellular mechanisms underlying the pathogenesis of AS, which are mainly related to altered synaptic plasticity (Bi et al., 2016). For instance, AS mice display a decreased number and size of dendritic spines in neurons across various brain regions, including the cerebellum, hippocampus and visual cortex (Dindot et al., 2007; Sun, Zhu, et al., 2015; Yashiro et al., 2009). Notably, recent findings revealed that in AS hippocampal neurons, the loss of UBE3A is associated with 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).

Another example of altered synaptic plasticity in AS is LTP impairment (Baudry et al., 2012). One of the first identified Ube3a target proteins in mammalian brain (Greer et al., 2010) is Arc (activity-regulated cytoskeletal protein), a protein known to be ubiquitinated and degraded in an Ube3a-dependent manner, and also modulated by Ube3a at the transcriptional level (Kühnle et al., 2013). Arc facilitates the internalization of the AMPA receptors (AMPAR), leading to reduction of AMPAR-mediated synaptic transmission. Consequently, the LTP impairment in AS may be attributed to increased Arc levels resulting from Ube3a absence (Rial Verde et al., 2006). The induction and maintenance of LTP rely on BDNF-induced signaling through its receptor, TrkB, which interacts with the postsynaptic density protein-95 (PSD-95) (Cao et al., 2013). In AS mice, characterized by elevated levels of Arc, PSD-95 is abnormally sequestered by Arc, its recruitment by TrkB is impaired, and consequently BDNF signaling is altered, contributing to LTP impairment (Cao et al., 2013).

It is noteworthy that both neuronal connectivity and LTP, which are altered mechanisms in AS, are affected by 5-HT7R stimulation. Indeed, activation of 5-HT7R has been shown to enhance dendritogenesis and increase neurite outgrowth (Speranza et al., 2015, 2017). Regarding LTP, 5-HT7R stimulation increases the phosphorylation of the GluA1 subunit of AMPA receptors, facilitating AMPA receptor-mediated neurotransmission (Andreetta et al., 2016). In addition, activation of 5-HT7R has been shown to promote an increase in TrkB receptor expression and phosphorylation, thereby affecting

BDNF signaling (Samarajeewa et al., 2014). Based on these considerations, modulation of 5-HT7R signaling may offer a potential avenue for rescuing the molecular mechanisms that are disrupted in AS.

2.4 Alterations of synaptic plasticity in neurodegenerative diseases

Neurodegenerative diseases are incurable and debilitating conditions caused by progressive neuronal loss. Mounting evidence suggests that synaptic plasticity mechanisms are also altered in several neurodegenerative diseases, including Alzheimer's disease (AD) (Battaglia et al., 2007; Cefaliello et al., 2020; Shao et al., 2011), Parkinson's diseases (PD) (Bagetta et al., 2010), and Huntington's disease (HD) (Orth et al., 2010). The synaptic plasticity mechanisms that were found altered in neurodegenerative diseases include BDNF signaling (Azman & Zakaria, 2022; Gao et al., 2022), synaptic transmission (Smith-Dijak et al., 2019), as well as local protein synthesis (Cefaliello et al., 2020). Using an AD animal model, it was demonstrated that the profile of newly synthesized proteins in AD mice synaptosomes was different from that in controls, and that the amyloid precursor protein (APP) is in part locally translated in the synaptosomal fraction (Cefaliello et al., 2020). In view of the fact that exposure of axons to β-amyloid activates local protein synthesis (Baleriola et al., 2014; Walker et al., 2018), the finding that APP itself can be locally synthesized raises the possibility that the effect of β-amyloid is amplified by a positive feedback loop provided by local expression and processing of APP (Perrone-Capano et al., 2021). In HD, mutant huntingtin protein (HTT) was found to impair axonal transport leading to

synaptic and axonal dysfunction, which precedes neuronal loss (Li & Conforti, 2013; Taran et al., 2020; Vitet et al., 2020).

Interestingly, exosomes were also demonstrated to be implicated in neurodegenerative disorders (Schloesser et al., 2008; Stephan et al., 2006). In particular, exosomes isolated from the brains of AD patients contain β-amyloid oligomers, and are able, in vitro, to transfer these oligomers from neuron to neuron (Sardar Sinha et al., 2018). Thus, it is possible to hypothesize that exosomes may contribute to spread degeneration in the brain.

2.4.1 Progressive myoclonic epilepsy type 1

Progressive myoclonic epilepsy type 1 (EPM1 or Unverricht-Lundborg disease OMIM 254800) is an autosomal recessive neurodegenerative disease. EPM1 is a rare disorder but represents the most common form of progressive myoclonic epilepsy (Kälviäinen et al., 2008). Clinical manifestations of the disease are observed in late childhood or adolescence, with onset between 6 and 16 years of age. Symptoms of EPM1 are myoclonic seizures and, less frequently, tonic-clonic seizures that become more severe and frequent with age (Kälviäinen et al., 2008). Individuals affected by EPM1 exhibit emotional lability, depression, and a gradual decline in intellectual performance (Lehesjoki & Kälviäinen, 1993). EPM1 is mainly caused by loss-of-function mutations in the cystatin B (CSTB) gene due to an expansion of an unstable dodecamer in the gene promoter (Joensuu et al., 2008; O'Brien et al., 2017). In the majority of patients, the mutation is present in a homozygous or heterozygous manner, along with an allele carrying a point mutation (Canafoglia et al., 2012; Lafrenière et al., 1997; Lalioti et al., 1997; Virtaneva

et al., 1997). The severity of the disorder correlates with the amount of functional CSTB (Joensuu et al., 2008; O'Brien et al., 2017). Knock-out (KO) mice for CSTB exhibit some of the human EPM1 phenotypes, and display apoptosis of granule cells in the cerebellum, associated with progressive ataxia and myoclonic seizures (Pennacchio et al., 1996, 1998), suggesting that CSTB plays a neuroprotective role. Accordingly, inducing epileptic activity in wild-type (WT) rats results in a transient increase in CSTB expression, counteracting the apoptotic mechanism (D'amato et al., 2000). Furthermore, CSTB deficiency in EPM1 mice has been linked to neuroinflammation, with early microglial activation associated with functional and morphological alterations preceding astrocyte activation, neuronal loss, and onset of myoclonus (Körber et al., 2016; Okuneva et al., 2015; Tegelberg et al., 2012). In line with this, CSTB-KO mouse brains display inflammation and early synaptic impairment (Joensuu et al., 2014).

2.4.2 Cystatin B and synaptic plasticity

Since the absence of CSTB is the primary cause of EPM1, the role of this protein in the nervous system has been widely investigated. CSTB is a protein inhibiting cysteine proteases, particularly cathepsin B, and is ubiquitously expressed in most cell types and tissues (Joensuu et al., 2008; Rispoli et al., 2013). Since the genetic removal of the target cathepsins in CSTB-KO mice does not fully rescue the pathological phenotype of these animals (Houseweart et al., 2003), it has been hypothesize that CSTB plays additional roles other than inhibiting cathepsins.

In the nervous system, CSTB has been identified in cortical neurons, in the dentate gyrus of the hippocampus and in the rat cerebellum (Riccio et al., 2005), where its expression is finely regulated during development. Indeed, during embryogenesis, it is present in granule cells, while in adults it is found in Purkinje cells (Di Giaimo, 2002; Riccio et al., 2005). Additionally, CSTB is also expressed in radial glial cells, while in the cerebellum, only oligodendrocyte progenitors express the protein (Di Giaimo, 2002). A similar situation is found in humans, where the distribution of CSTB in cerebellar cells is restricted to Purkinje cells and Bergmann fibers (Riccio et al., 2005). CSTB has been identified in various subcellular compartments (Alakurtti et al., 2005; Brännvall et al., 2003; Calkins et al., 1998), where its localization depends on the differentiation state, being predominantly nuclear in proliferating cells, but present in both the nucleus and the cytoplasm in differentiated cells. This cellular localization does not correlate with that of cathepsin B, except to a small extent in the extracellular matrix (Alakurtti et al., 2005; Joensuu et al., 2007), confirming alternative roles for CSTB in addition to the protease inhibition.

CSTB protects neurons from oxidative stress (Lehtinen et al., 2009) and prevents cerebral apoptosis (Pennacchio et al., 1998), suggesting that it contributes to neuronal plasticity. Accordingly, it has been demonstrated that CSTB is locally synthesized in synaptic areas of the rat brain (Penna et al., 2019). The link between CSTB and synaptic plasticity has been further supported by a number of experimental data showing that (i) CSTB-KO mice display altered abundance of ribonucleoproteins and ribosomal subunits in synaptic regions (Gorski et al., 2020); (ii) CSTB deficiency leads to alterations in GABAergic signaling (Gorski et al., 2020; Joensuu et al., 2014); (iii) CSTB is

involved in interneuron recruitment during neurogenesis, and in modulating the extracellular environment (Di Matteo et al., 2020); (iv) CSTB is secreted by nerve terminals in a depolarized-dependent way (Penna et al., 2019); (v) CSTB contributes to autophagy and vesicular trafficking in astrocytes (Polajnar et al., 2014).

CSTB aggregates have been detected in association with senile plaques of Alzheimer's and Parkinson's disease patients (Ii et al., 1993; Žerovnik, 2017), and a mouse model of amyotrophic lateral sclerosis displays altered levels of CSTB in the spinal cord (Rosen et al., 1993; Wootz et al., 2006), Since these pathologies are characterized by deregulation of synaptic plasticity (Cefaliello et al., 2020; Gulino, 2023; Skaper et al., 2018), these results confirm that CSTB is involved in the altered mechanisms of synaptic plasticity in neurodegenerative diseases.

3. AIMS

Synaptic plasticity, the dynamic ability of the nervous system to adapt in response to external and internal stimuli, involves diverse cellular and molecular mechanisms, including protein synthesis in nerve terminals and EVs release. Alterations in synaptic plasticity have been demonstrated to be a common feature of several neurodevelopmental and neurodegenerative disorders. Thus, investigating synaptic plasticity mechanisms in physiological and pathological conditions could be a promising strategy for developing novel therapeutic treatments for diverse neuropathologies (Bliss et al., 2014).

The first aim of this work was to study alterations of synaptic plasticity in two animal models of neurodevelopmental diseases, i.e. Autism Spectrum Disorder and Angelman Syndrome, and in a human model of neurodegenerative diseases, the Progressive Myoclonic Epilepsy type 1. Since deficits at the synaptic level are a common feature of these pathologies, we hypothesised that at least some of the molecular mechanisms involved in their pathogenesis, may be similar. In particular, we focused our attention on the synaptic system of protein synthesis and on the secretion of extracellular vesicles, using brain synaptosomes as *in vitro* model. We also used synaptosomes prepared from human cerebral organoids (hCOs) derived from EPM1 patients and controls. This approach allowed us to confirm the validity of hCOs as a model for studying synaptic plasticity mechanisms and offered a valuable opportunity to extend our results to a human model.

If different neuropathologies share the same altered molecular mechanisms, it is intriguing to identify a treatment able to reverse some of these alterations. From this point of view, **our second aim** was to investigate the possible beneficial effects of stimulation of the serotonin receptor 7, using the

selective agonist LP-211. Indeed, serotonin modulates various physiological aspects of the nervous system, and the 5-HT7R has been demonstrated to play a crucial role in neuronal plasticity (Crispino et al., 2020). Accordingly, disruption of its signaling pathways contributes to impaired neuronal connectivity and cognitive dysfunctions (Crispino et al., 2020).

4. MATERIALS AND METHODS

4.1 Animals

Wistar male rats (Charles River Laboratories, Calco, Lecco, It), of about 3 months of age, were kept in the animal house at the Department of Biology, University of Naples Federico II, Italy.

BTBR T + tf/J (BTBR, The Jackson Laboratory, Bar Harbor, ME, USA), were used as an animal model for autism spectrum disorder, and their control C57Bl/6J mice of about 30 days of age were kept in the animal care facility at the Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Italy.

C57BL/6J mouse embryos (E18) were kept in the animal facility of the Max Planck Institute of Psychiatry, Munich, Germany. The embryonic day was defined based on the day of vaginal plug, which was considered as embryonic day 0 (E0).

All animal handling procedures were performed in accordance with European Union guidelines.

Ube3A$^{tm1Alb/J}$ mice (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 described (Baudry et al., 2012). AS and WT mice were used at about 2 to 3 months of age. WT and AS pups were used for neuronal primary cultures. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Western University of Health Sciences (Pomona, California), in accordance with the National

Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Animals were kept with food and water ad libitum in a room with controlled temperature and humidity, and a 12-h light-dark regimen.

4.2 Generation of human cerebral organoids

Reprogrammed induced pluripotent stem cells (iPSCs) from 2 control and 2 EPM1 patient-derived cell lines were used to generate human cerebral organoids (hCOs), as previously described (Lancaster 2013, 2014, Di Matteo et al., 2020). The two EPM1 patients carried two different mutations for the pathology: E1 is bearing a homozygous amplification in the CSTB promoter, while E2 is heterozygote for the dodecamer repeat and has a point mutation leading to skipping of exon 2. Both mutations lead to pathological low level of functional CSTB. Briefly, iPSCs were dissociated into single cells using StemPro Accutase Cell Dissociation Reagent (A1110501, Life Technologies) and plated at the concentration of 9000 single iPSCs/well into low-attachment 96-well tissue culture plates in hES medium (DMEM/F12GlutaMAX supplemented with 20% Knockout Serum Replacement, 3% ES-grade FBS, 1% nonessential amino acids, 0.1 mM 2-mercaptoethanol, 4 ng/mL bFGF, and 50 μM Rock inhibitor Y27632) in order to form embryoid bodies (EBs). On day 6, EBs were transferred into low-attachment 24-well plates in NIM medium (DMEM/F12GlutaMAX supplemented with 1:100 N2 supplement, 1% nonessential amino acids, and 5 μg/mL heparin) and cultured for additional 6 days. On day 12, EBs were embedded in Matrigel (Corning, 354,234) drops and then transferred in 10-cm tissue culture plates in NDM minus A medium

(DMEM/F12GlutaMAX and Neurobasal in ratio 1:1 supplemented with 1:100 N2 supplement 1:100 B27 without vitamin A, 0.5% nonessential amino acids, insulin 2.5 µg/mL, 1:100 antibiotic–antimycotic, and 50 µM 2-mercaptoethanol) in order to form hCOs. On day 4, after Matrigel embedding, hCOs were transferred in 10-cm dishes into an orbital shaker and cultured until analysis in NDM plus A medium (DMEM/F12GlutaMAX and Neurobasal in ratio 1:1 supplemented with 1:100 N2 supplement 1:100 B27 with vitamin A, 0.5% nonessential amino acids, insulin 2.5 µg/mL, 1:100 antibiotic–antimycotic, and 50 µM 2-mercaptoethanol). hCOs were kept on a shaker at 37 °C, 5% CO_2 and ambient oxygen level with NDM plus A medium, with changes of medium every 3–4 days. hCOs were analyzed at different months after the initial plating of the cells as indicated in the "Results" section and figures.

4.3 Synaptosomal fraction

4.3.1 Isolation of synaptosomal fraction from hCOs and rodents cerebral cortex

Synaptosomes were prepared from different samples: i) a pool of 20–40 organoids, ii) cerebral cortex dissected from 3 months old rats (n=3), and iii) cerebral cortex of mouse embryos at embryonic day 18 (E18, n=5), iv) cerebral cortex dissected from 30 days old BTBR and WT mice (n=5-6), v) cerebral cortex dissected from 3 months old of AS and WT mice (n=5-7). Synaptosomes were prepared as previously described (Cefaliello et al., 2020; Eyman et al., 2007; Penna et al., 2019). Briefly, cerebral cortices or hCOs were resuspended

in nine volumes of cold isotonic medium (IM: 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4) and homogenized with a Dounce homogenizer. An aliquot of the homogenate was dissolved in RIPA buffer (50 mM Tris-HCl pH 8.8, 150 mM NaCl, 0.1% SDS, 0.5% NP-40, 0.5% DOC; protease and phosphatase inhibitor cocktail, Sigma-Aldrich) for subsequent analysis. The remaining homogenate was centrifuged at 2,000 x g for 1 min, 4°C. The sediment was resuspended in the same volume of IM and centrifuged again under the same conditions to remove the sediment containing nuclei, cell debris and other particulates. The two supernatants were combined and centrifuged at higher speed (23,000 x g for 4 min, 4°C) to obtain a pellet that was washed in the same volume of IM and centrifuged again as described above. The obtained pellet, named P2 fraction or crude synaptosomal fraction, contains mitochondria, myelin and synaptosomes. Synaptosomes from adult rats and mouse embryo cortices were further purified by discontinuous Ficoll gradient. The P2 fraction (1 mL with protein concentration of 3.2 mg/mL) was stratified on a discontinuous gradient 5-13% Ficoll in IM (2 mL each) by centrifuging at 45,000 x g for 45 min at 4°C. The purified synaptosomes were collected from the interface, diluted with nine volumes of IM and sedimented at 23,000 x g for 20 min, 4°C. The pellet was homogenized in IM and used for subsequent analyses. The protein content of both homogenate and synaptosomal fractions was determined by BIO-RAD method using bovine serum albumin (BSA) as the standard protein.

4.3.2 Incubation and isolation of secreted proteins from synaptosomes

Synaptosomal fraction from hCOs and rodents cortices was incubated in ringer medium (90 mM NaCl, 3 mM KCl, 2 mM MgCl2, 1 mM CaCl2, 1 mM glucose, 100 mM sucrose, 30 mM Tris-HCl, pH 7.5), or in depolarizing medium (43 mM NaCl, 50 mM KCl, 2 mM MgCl2, 1 mM CaCl2, 1 mM glucose, 100 mM sucrose, 30 mM Tris-HCl, pH 7.5) or in depolarizing medium without calcium (43 mM NaCl, 50 mM KCl, 2 mM MgCl2, 1 mM glucose, 103 mM sucrose, 30 mM Tris-HCl, pH 7.5). Synaptosomes were incubated with LP-211 (100 nM), or DMSO (0,1%, vehicle of LP-211), or BAPTA-AM (2 µM) or ionomycin (100 nM) as indicated in the "Results" section. After 1 h incubation at 37°C, samples were cooled on ice and centrifuged at 23,000 x g for 7 min, 4°C. The resulting pellet, containing post-incubation synaptosomes, was resuspended in RIPA buffer. The supernatant, containing the secreted proteins (here defined as secretome), was further centrifuged at 100,000 x g for 2h, 4°C. The pellet, containing the extracellular vesicles (EVs) (Théry et al., 2006), was resuspended in RIPA buffer for Western Blot analysis (WB), or in PBS for immunostainings. The supernatant, containing the soluble proteins (SF, soluble-protein fraction) was concentrated by Amicon Centrifugal Filter Devices with a cut-off of 10 kDa (Merck-Millipore).

4.3.3 SUrface and SEnsing of Translation (SUnSET) in the presence of LP-211

DIV7 hippocampal neurons were incubated for 30 minutes with 1 µM puromycin (p8833 sigma), to detect newly synthetized protein, and 100 nM LP-211, to assess the effect of 5-HT7R activation on protein synthesis. Cells

were then rinsed with PBS and subsequently lysed in RIPA buffer for western blot analysis.

Synaptosomes from rodent cortices were preincubated at 37°C for 15 minutes with 15 µM anisomycin (Aniso, 1290 Tocris) and 100 nM SB-269970 (1612 Tocris) when required. For all samples (with or without preincubation) the reaction started by adding the synaptosomal fraction (300 µg/ml) to the incubation medium containing 20 µM puromycin, 15 µM anisomycin (when required), 100 nM LP-211, or 100 nM SB-269970. After 1 h incubation at 37 °C, the reaction was stopped by cooling the samples in ice. Synaptosomes were collected by centrifugation at 23,000 g, 7 min, 4 °C. The pellet, containing synaptosomal proteins, was resuspended in RIPA Buffer, 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.

4.4 Immunostaining of synaptosomes and EVs from mouse embryos

Purified synaptosomes from E18 mice brain cortex were fixed in 4% Paraformaldehyde (PFA) for 10 min and permeabilized with 0.3% Triton X-100 for 5 min. The samples were then incubated in the blocking solution: 0.1% Tween, 10% Normal Goat Serum (Biozol, LIN-ENG9010-10) for 2 h at 4°C. The primary antibodies were diluted with blocking solution prior to the overnight incubation at 4°C: synaptophysin (SYP, 1:1.000, Millipore, AB9272), cystatin B (CSTB, 1:500, Antikoerper AbIN271833), CD81 (1:500, Santa Cruz sc-166029). The synaptosomes were then washed three times in PBS by centrifugation (23,000 x g, 7 min at 4°C), and the collected pellet was incubated with secondary antibodies (Alexa Fluor® 488 Goat Anti-Rabbit IgG (H + L) (1:1,000,

Life-Technologies A11008), Alexa Fluor® 546 Goat Anti-Mouse IgG (H + L) (1:1,000, Life-Technologies A-11003), for 1h at 4°C with gentle shaking. After centrifugation, the pellet was washed 3 times with PBS and finally resuspended in 10 µL of PBS before mounting on microscope slides to be analyzed with a Leica laser-scanning microscope.

EVs released by synaptosomes from E18 mice brain cortex were stained as previously reported (Mondal et al., 2019), with slight modifications. Briefly, the PBS suspension of EVs was added to an equal volume of 20% PEG1000 (Sigma, 92897), incubated for 1h at 4°C with gentle shaking, and centrifuged at 3,000 x g for 5 min. The pellet, containing EVs, was resuspended in PBS and permeabilized with 0.001% Triton X-100 for 5 min. The resuspended EVs was added to an equal volume of 20% PEG and centrifuged at 3,000 x g for 5 minutes and washed 3 times in 10% PEG. The EVs pellet was resuspended in PBS and incubated overnight with primary antibodies (CSTB 1:1,000 Antikoerper AbIN271833, CD81; 1:500 sc-166029 Santa Cruz Biotechnology) at 4°C with gentle shaking. Samples were washes 3 times with PEG 10% in PBS at 3,000 x g for 5 min, 4°C. Pellets were resuspended in PBS, and the secondary antibodies Alexa Fluor® 488 Goat Anti-Rabbit IgG (H + L) (1:1,000, Life-Technologies A11008), and Alexa Fluor® 546 Goat Anti-Mouse IgG (H + L) (1:1,000, Life-Technologies A-11003) were incubated for 1h at 4°C with gentle shaking. The samples were washed 3 times with PBS, purified from the unbound antibodies using Sephadex G-25 column (Sigma G2580), resuspended in 10 µL of PBS and mounted on a microscope slide. Immunostained sections were analyzed with a Leica SP8 confocal laser-scanning microscope. Only signals whose dimensions do not exceed 1 µm

were analyzed, according to Mondal et al 2019. Colocalization of two proteins in the same synaptosome or EV samples was assessed by merging the different fluorescent signals on the same confocal plane. Yellow color indicates the overlap of the red and green signals.

4.5 Gel Electrophoresis and Western Blot analysis

Aliquots of homogenates and synaptosomal fractions were resuspended in RIPA Buffer. The protein samples were denatured at 100 °C for 5 min in the sample buffer (60 mM Tris-HCl pH 6.8, 10% Glycerol, 2% SDS, 100 mM DTT 0.1% bromophenol blue), separated in 10%-15% SDS-PAGE and were transferred to PVDF membranes (Merck-Millipore). The same amount of proteins was loaded in each lane of the gel. Western blot analysis was performed as previously reported (Penna et al., 2019) with the primary antibodies indicated in table 1. After several washings in TBST, membranes were incubated with secondary antibody against rabbit (1:20,000, A0545, Sigma-Aldrich) or mouse (1:20,000, NA931, GE Healthcare) IgG linked to horseradish peroxidase. Signals were visualized by chemiluminescence (ECL, Millipore) on X-ray film (X-Ray Film, Fujifilm).

Western blots of AS mice and respective controls were performed accordingly to published protocols (Sun, Zhu, et al., 2015) Briefly, samples were resuspended in RIPA buffer (50 mM Tris-HCl pH 8.8, 150 mM NaCl, 0.1% SDS, 0.5% NP-40, 0.5% DOC; protease and phosphatase inhibitor cocktail, Sigma-Aldrich) and the protein concentration of the samples was determined by the BCA Protein Assay (Thermo Fisher). The same amount of proteins was separated by SDS-PAGE and transferred to a PVDF membrane (Millipore).

After blocking with 3% BSA for 1 h, membranes were incubated with specific antibodies overnight at 4 °C followed by incubation with secondary antibodies (IRDye secondary antibodies) for 2 h at room temperature. Antibody binding was detected using the Odyssey imaging system.

Table 1.

5-HT7R	1: 1,000	NB100-56352 Novusbio
p-ERK	1:1,000	9101 Cell Signaling
ERK	1:1,000	sc-271269 Santa Cruz
p-P70	1:1,000	9234 Cell Signaling
P70	1:1,000	2708 Cell Signaling
p-AKT	1:1,000	4060 Cell Signaling
AKT	1:1,000	9272 Cell Signaling
Puromycin	1:500	12D10 Millipore
eIF2B2	1:2,000	HPA005841 Sigma-Aldrich
eIF4G2	1:1,000	HPA016965 Sigma-Aldrich
p-eIF4E	1:10,000	Cell Signaling
eIF4E	1:1,000	9742 Cell Signaling
Synaptophysin (SYP)	1:1,000 hCOs; 1:200,000 rodents	AB9272 Millipore
Syntaxin (STX)	1:500 hCOs; 1:20,000 rodents	E-AB-33012 Elabscience
Synaptotagmin 1/2 (SYT)	1:1,000	E-AB-33005 Elabscience

CD81	1:500	Sc-166029 Santa Cruz
CD82	1:500	sc-518002 Santa Cruz
CD9	1:500	sc-13118 Santa Cruz
CSTB	1:2,000	Antikoerper AbIN271833
TAU	1:20,000	E-AB-33036 Elabscience
β-actin (ACTB)	1:2,000	612656 BD Biosciences
GAPDH	1:400,000	CB1001 Sigma-Aldrich
α-tubulin	1:600,000	Ab47074 Abcam

4.6 Hippocampal neuronal cultures

Neuronal cultures were prepared from hippocampus of wild-type (WT) or Ube3am-/p+ mice. Genotyping was carried out by polymerase chain reaction (PCR) of mouse tail DNA as previously described (Baudry et al., 2012). Hippocampal neurons were prepared from P1-P3 mouse as described (Sun, Liu, et al., 2015). Briefly, hippocampi were dissected and digested with papain (2 mg/ml, Sigma P4762) for 20 min at 37°C. Dissociated cells were filtered with a cell strainer (Fisherbrand 22363548) and plated onto poly-D-lysine-coated coverslips in 24-well plates at a density of 10-15 x 10^3 cells/cm^2 in Neurobasal medium A (GIBCO 10888-022) supplemented with 5% FBS, SM1 (StemCell Technologies), Glutamine and pen/strep and kept at 37 °C under 5% CO_2. Whole medium was replaced with fresh culture medium (Brayn Physiology, StemCell Techonolgies 05790) supplemented with SM1 and pen/strep at DIV1

and then every 7 d. On DIV13 cells were transfected with GFP adenovirus (SantaCruz sc-108084). On DIV18-19-20 cells were stimulated with 100 nM LP-211, 100 nM SB-269970 or a combination of both.

4.6.1 Immunofluorescence of primary cultures

Cultured hippocampal neurons were fixed in 2% paraformaldehyde (PFA)/10% sucrose for 15 min at 37 °C, transferred to 0.05% Triton X-100/PBS for 5 min at 4 °C, and then 0.02% Tween-20/PBS for 2 min at 4 °C. Coverslips were washed twice with ice cold PBS and incubated 1 hr in 3% BSA/PBS at room temperature. For staining of F-actin, cells were incubated with anti-F-actin (1:500, R415, invitrogen) in 3% BSA/PBS overnight at 4 °C. Coverslips were then washed twice with ice cold PBS for 10 min each and then incubated with secondary antibodies (Alexa Fluor-633 anti-mouse, 1:200) for 2 h at room temperature. Coverslips were then washed four times with ice cold PBS for 10 min each and mounted on glass slides using VECTASHIELD mounting medium with DAPI (Vector Laboratories). Images were acquired using a Zeiss LSM 880 confocal laser-scanning microscope. The staining was visualized in GFP-expressed neurons. Mean fluorescence intensity (MFI) was calculated over a specific region of interest, and background staining of the sections was measured and subtracted from the total signal to obtain the specific signal.

4.6.2 Image analysis and quantification

Images were acquired using a Zeiss LSM 880 with Airyscan confocal laser-scanning microscope with a 60 X objective. Images for all groups in a particular

experiment were obtained using identical acquisition parameters and analyzed using ImageJ software (NIH). All immunostaining studies were performed in three independent experiments.

4.6.3 Dendritic spine analysis

The number of spines located on randomly selected dendritic branches was counted manually. Spine density was calculated by dividing the number of spines on a segment by the length of the segment and was expressed as the number of spines per mm of dendrite. About 15 different dendritic branches of 20 µm in length, derived from about 3 different neurons, were analyzed and averaged for each animal. The analysis was performed on 3 animals for each experimental condition.

4.7 Neuronal progenitor cells (NPCs)

4.7.1 Neuronal differentiation from NPCs

Neuronal cultures were obtained from NPCs of 2 control and 2 patient-derived cell lines (Di Matteo et al., 2020) as previously described (Gunhanlar et al., 2018). Neurons were cultured for 8 weeks in the neuronal differentiation medium (Neurobasal medium: 1% N2 supplement; 2% B27 w/o vitamin A; 1% MEM-NEA; 1,25 µg/µL laminina; 1% penicillin/streptomycin, with addition of fresh 20 ng/mL BDNF, ProSpec Bio, Rehovot, Israel, 20 ng/mL GDNF, ProSpec Bio, 200 µM cAMP, Sigma-Aldrich, 1 µM ascorbic acid, Sigma-Aldrich). From week 1 to 4 the medium was changed every other day; from week 4 to 10 only half of the medium was replenished.

4.7.2 3D reconstruction of neurons for morphological analysis

Mature neurons, obtained from NPCs differentiation, were used for the morphological analysis. A sparse labeling of cells with GFP was obtained by lipofection of CMV-EGFP plasmid using Lipofectamine 3000 Reagent following the manufacturer instructions (Thermo Fisher Scientific, L3000001). 2 days after lipofection, neuronal cultures were fixed and immunostained as described above. As primary and secondary antibodies were also used: GFP (GFP-1020, 1:1,000, Aves Lab), Alexa Fluor® 488 Goat Anti-Chicken IgY (H + L) (1:1,000, Life-Technologies A11039). Neurons were visualized using a Leica SP8 confocal laser-scanning microscope with 40x objective. Z-projections with a Z-Step Size of 1 µm were taken to obtain 3D image. Confocal images were acquired with the Neurolucida Software (MBF Bioscience, Neurolucida version 2017.03.3, 64-bit), and the subsequent tracing was performed in 3D. For the analysis of the reconstructed neurons, Neurolucida Explorer (MBF Bioscience, Neurolucida version 2017.02.9) was used. The "Branched Structure Analysis" tool was used for the neuron summary analysis and the individual process analysis. Furthermore, neuronal complexity was assessed using the Sholl analysis tool. The concentric circles were traced using a radius increment of 10 µm.

4.8 Mass Spectrometry

Aliquots of proteins from homogenate (h) and synaptosomal fractions (syn) of 1.5 months old control and EPM1 hCOs were used for mass spectrometric analysis, as previously described (Di Matteo et al., 2020). For each condition,

equal amounts of proteins (80 µg in 100 µL of 100 mM Triethylammonium bicarbonate) were digested with trypsin and labeled with the following TMT isobaric tags according to the procedure described elsewhere (Citores et al., 2020): 128C and 127C for syn CTR and h CTR samples, and 130N and 129N for syn EPM1 and h EPM1 samples, respectively. TMT-labeled samples were then diluted with 2% TFA to the final protein concentration of 0.5 µg/µL for the LC-MS/MS analyses. Aliquots of TMT-labeled peptides (2.5 µg) were analyzed by high-resolution nanoLC–tandem mass spectrometry using a Q-Exactive Orbitrap mass spectrometer equipped with an EASY-Spray nanoelectrospray ion source (Thermo Scientific, Rockford, Illinois, USA), coupled to a Thermo Scientific Dionex UltiMate 3000 RSLCnano system (Thermo Scientific, Rockford, Illinois, USA) as reported elsewhere (Russo et al., 2019). The acquired raw files were analyzed with the Thermo Scientific Proteome Discoverer 2.1 software (Thermo Scientific, Rockford, Illinois, USA) using the SEQUEST HT search engine. The HCD MS/MS spectra were searched against the Homo sapiens Uniprot database (release 2019_11, 20380 entries) by using the following parameters: trypsin (Full) as digestion enzyme with two missed cleavage sites; mass tolerances: 10 ppm and 0.02 Da, for precursor and fragment ions, respectively; dynamic modification: methionine oxidation (+15.995 Da); static modifications: carbamidomethylation of cysteine (+57.021 Da) and the TMT label on lysines and the N-terminus (229.1629). False discovery rates (FDRs) for peptide spectral matches (PSMs) were calculated and filtered using the Percolator node in Proteome Discoverer that was run with the following settings: Maximum Delta Cn 0.05, a strict target FDR of 0.01, a relaxed target FDR of 0.05, and validation based on q-value.

Protein identifications were accepted when the protein FDR was below 1% and when present in at least two out of three replicate injections with at least two peptides. Proteins with fold change values syn/h ≥ 1.2 and ≤ 0.8 were considered for bioinformatic analyses. Proteins identified by Mass Spectrometry were assigned to Gene Ontology (GO) biological process groups using STRING (string-db.org), version 11.5 with default parameters and in reference to the Homo sapiens as organism.

4.9 Statistical Analyses

All the statistical analyses in this study were performed using GraphPad Prism 8 software. Data were expressed as 'mean ± SEM' or 'mean ± SD' as indicated in the figure legends. Differences between groups were compared by t-test, one-way ANOVA with Tukey's post-test, nonparametric Mann-Whitney test or two-way ANOVA with Sidak's post-test as indicated in the Figure legends. Differences were considered statistically significant if $p < 0.05$.

5. RESULTS

5.1 5-HT7R stimulation decreases local protein synthesis in synaptosomes from rat brain

5-HT7R is strongly involved in synaptic plasticity and its deregulation is linked to several neurodevelopmental disease (Crispino et al., 2020). Our analyses revealed that 5-HT7R is expressed in rat brain cortex homogenates, but also in synaptosomal fractions (Fig. 2A). To investigate the pathways activated by synaptosomal stimulation of 5-HT7R we incubated synaptosomes with the selective 5-HT7R agonist, LP-211. In the presence of LP-211, levels of the phosphorylated form of ERK protein increased significantly (Fig. 2B), suggesting that the ERK pathway is selectively activated by 5-HT7R stimulation. Indeed, the phosphorylated form of P70 and AKT did not change significantly in presence of LP-211, indicating that their signaling pathways were not affected by 5-HT7R activation (Fig. 2C,D).

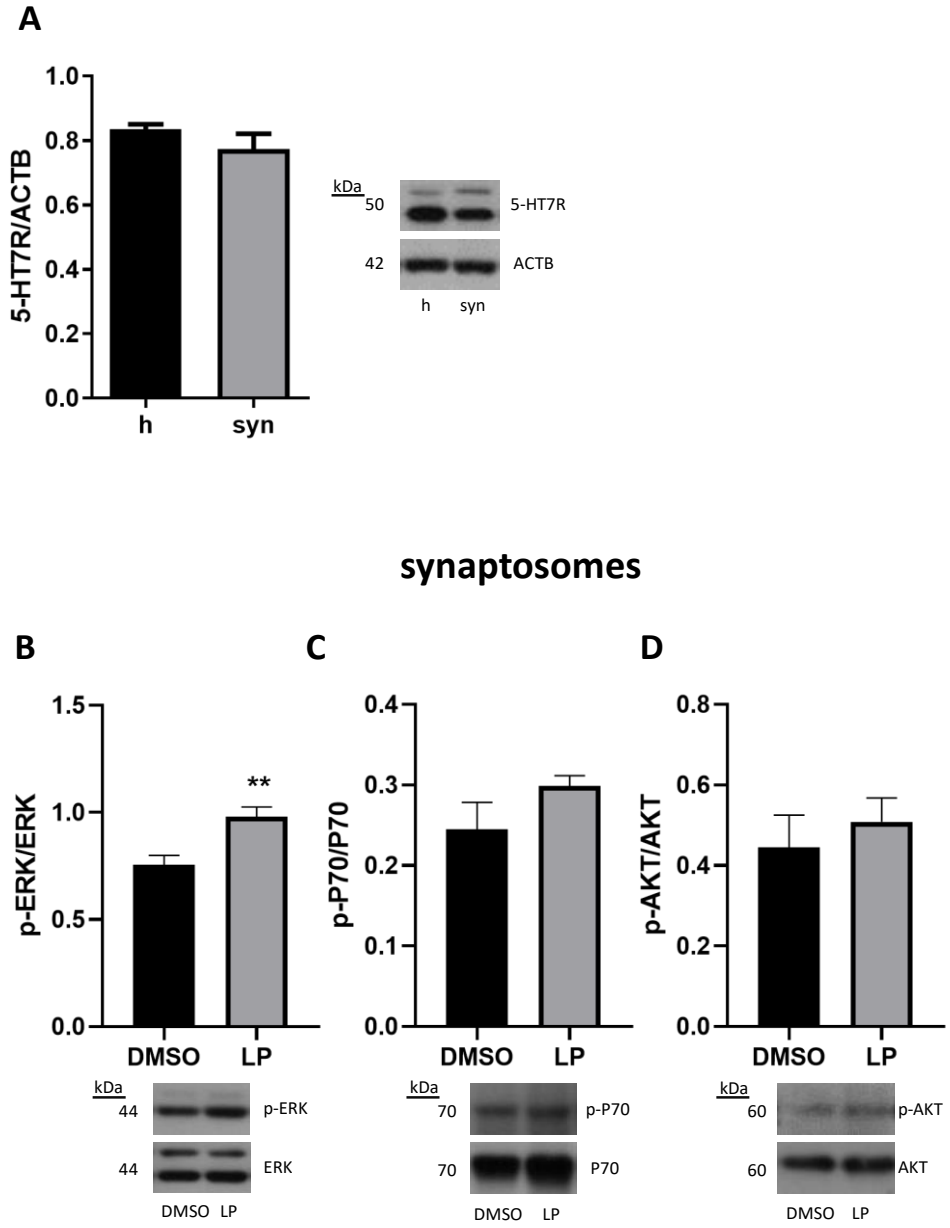


Figure 2: 5-HT7R protein levels and activation of ERK pathway following 5-HT7R stimulation in rat brain cortex synaptosomes. (A) *5-HT7R protein levels normalized to β-actin (ACTB) in the homogenate (h) and in the corresponding synaptosomal fraction (syn) from rat brain cortex. Representative images of*

*western blots analysis on the right. Protein levels of **(B)** p-ERK normalized on ERK, **(C)** p-P70 normalized on P70, and **(D)** p-AKT normalized on AKT, in synaptosomal fraction incubated with LP-211 (LP, 100 nM) or its vehicle (DMSO). Representative images of western blot analysis below the graphs. Molecular weight of each protein, in kDa, is indicated on the left. Data are presented as mean ± SEM. n=3-4. Statistically significant differences are based on unpaired t-test, ** p value< 0,01.*

To deepen our analysis of the involvement of 5-HT7R in synaptic plasticity, we studied the effects of 5-HT7R stimulation on the machinery of protein synthesis located in nerve terminals. To this aim, we analyzed the synaptic expression level of the eukaryotic initiation factor 2B2 (eIF2B2), which is a component of the protein synthesis machinery, which was previously demonstrated to be axonally synthesized (Kar et al., 2013). Our results showed a significant decrease in the expression levels of eIF2B2 in synaptosomes treated with LP-211, compared to the vehicle (Fig. 3A). These results suggest an inhibitory effect of 5-HT7R stimulation on synaptic protein synthesis, at least for some proteins. To confirm this hypothesis, we used the SUnSET method to label newly synthetized proteins in synaptosomes. The profile of puromycin-labeled proteins exhibited a significant decrease in the presence of anisomycin, an eukaryotic protein synthesis inhibitor (Fig. 3B), confirming that SunSET is an effective method to detect newly-synthesized proteins. When synaptosomes were incubated with LP-211 a significant decrease in the amount of newly synthetized proteins was detected, as compared to vehicle (Fig. 3B), confirming that the stimulation of 5-HT7R in the synaptic region decreases local protein synthesis.

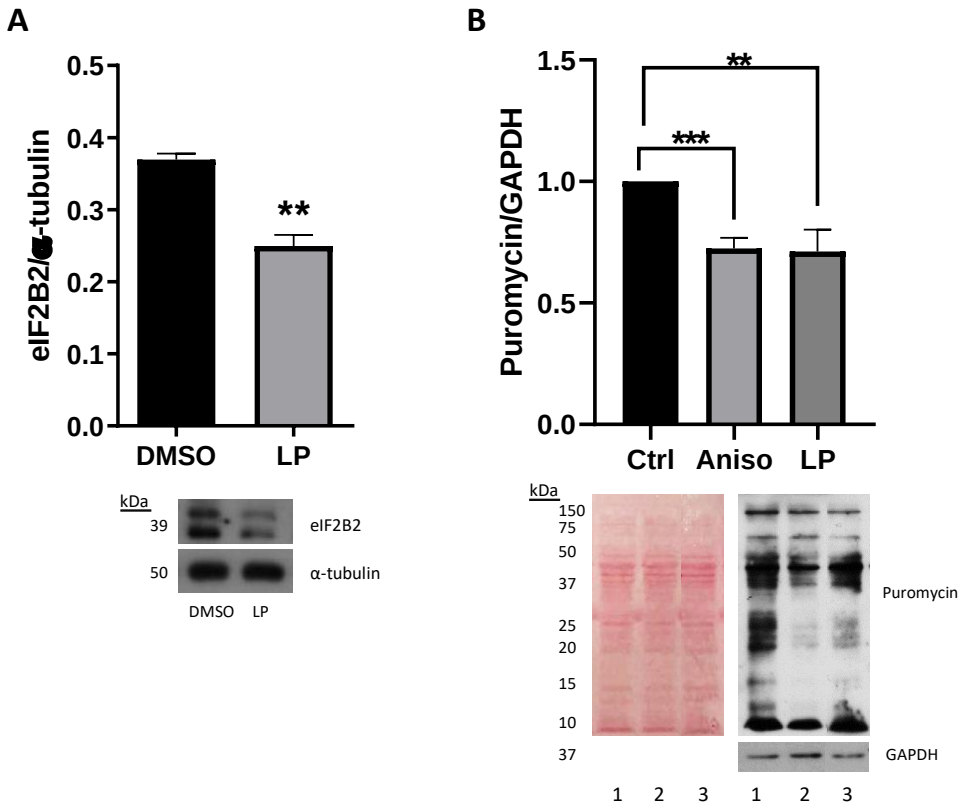


Figure 3: 5-HT7R stimulation induces a decrease in protein synthesis in rat brain cortex synaptosomes. (A) *Protein levels of the eukariotic Initiation Factor 2B2 (eIF2B2) normalized to α-tubulin in synaptosomes incubated with LP-211 (LP, 100 nM) or its vehicle (DMSO).* ***(B)*** *Levels of puromycinilated proteins normalized to GAPDH in synaptosomes incubated with anisomycin (aniso, 40 µM), or LP-211 (100 nM). Representative images of western blot analysis below the graphs. The red ponceau staining of membrane was used as a loading control, and is displayed below the graph in* ***(B)****. Molecular weight of each protein, in kDa, is indicated on the left. Data are presented as mean ± SEM. n=4-7. Statistically significant differences are based on unpaired t-test ** p value< 0,01, *** p value<0,001.1=Ctrl, 2=Aniso, 3=LP.*

5.2 Autism spectrum disorder (ASD) and alteration of synaptic plasticity

5.2.1 ASD mouse model displays alteration of 5-HT7R expression level in the synaptic area

ASD is a neurodevelopmental disorder whose symptoms are associated with several synaptic plasticity alterations (Hansel, 2019). Numerous findings correlate ASD defects with alteration of serotonergic signaling (Garbarino et al., 2019; Muller et al., 2016; Rodnyy et al., 2023), and in particular with 5-HT7R (Lee et al., 2021). We investigated the potential alterations in the synaptic area of BTBR mice, an ASD animal model, and we also analyzed the effects of 5-HT7R stimulation.

We first evaluated the synaptosomal enrichment of presynaptic proteins compared to the corresponding homogenate in both BTBR and WT mice (Fig. 4A-C). Our results showed that BTBR synaptosomes were particularly enriched in synaptophysin, a presynaptic vesicle marker, as compared to WT (Fig. 4A), while the enrichment in synaptotagmin 1/2 (Fig. 4B), a calcium sensor, and syntaxin (Fig. 4C), a t-SNARE protein, did not change in synaptosomal fraction of BTBR, as compared to WT.

We evaluated the expression levels of 5-HT7R in BTBR cerebral cortex homogenates (Fig. 4D) and in the corresponding synaptosomal fractions (Fig. 4E). Our results showed that the 5-HT7R level did not change in BTBR homogenates, as compared to WT (Fig. 4D), while it significantly decreased in BTBR synaptosomes, as compared to WT (Fig. 4E), indicating a specific alteration in synapses. We also detected a significant reduction of the ratio pERK/ERK in BTBR synaptosomes, as compared to the WT (Fig. 4F). Since ERK

is one of the molecular pathways associated with 5-HT7R activation, these data confirm the involvement of altered 5-HT7R signaling in ASD.

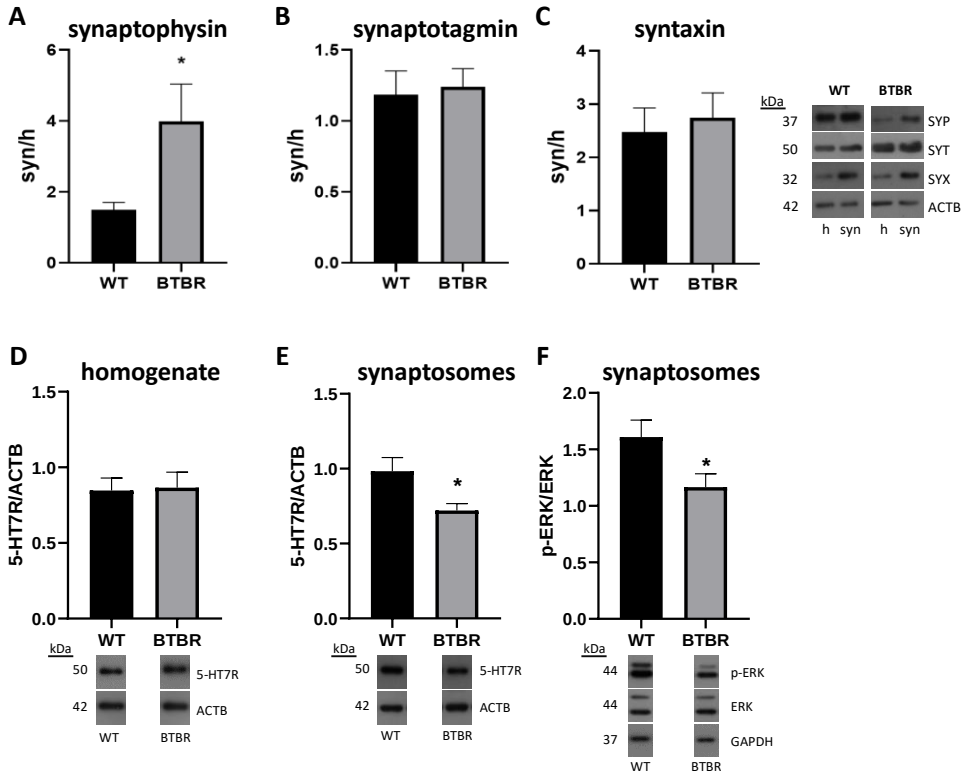


Figure 4: Synaptic protein enrichment, 5-HT7R and pERK protein levels in synaptosomes from cerebral cortex of BTBR and WT mice. *Enrichment of* ***(A)*** *synaptophysin (SYP),* ***(B)*** *synaptotagmin 1/2 (SYT) and* ***(C)*** *syntaxin (STX) normalized to β-actin (ACTB) in synaptosomes (syn) compared to homogenate (h) from WT and BTBR mice brain cortex. Representative images of western blot analysis on the right. 5-HT7R protein levels normalized to β-actin (ACTB) in* ***(D)*** *the homogenate and* ***(E)*** *synaptosomes from WT and BTBR mice brain cortex.* ***(F)*** *Protein levels of p-ERK normalized to ERK in synaptosomes from WT and BTBR mice brain cortex. GAPDH was used as loading control. Representative images of western blot analysis below the graphs. Molecular weight of each protein, in kDa, is indicated on the left. Data are presented as*

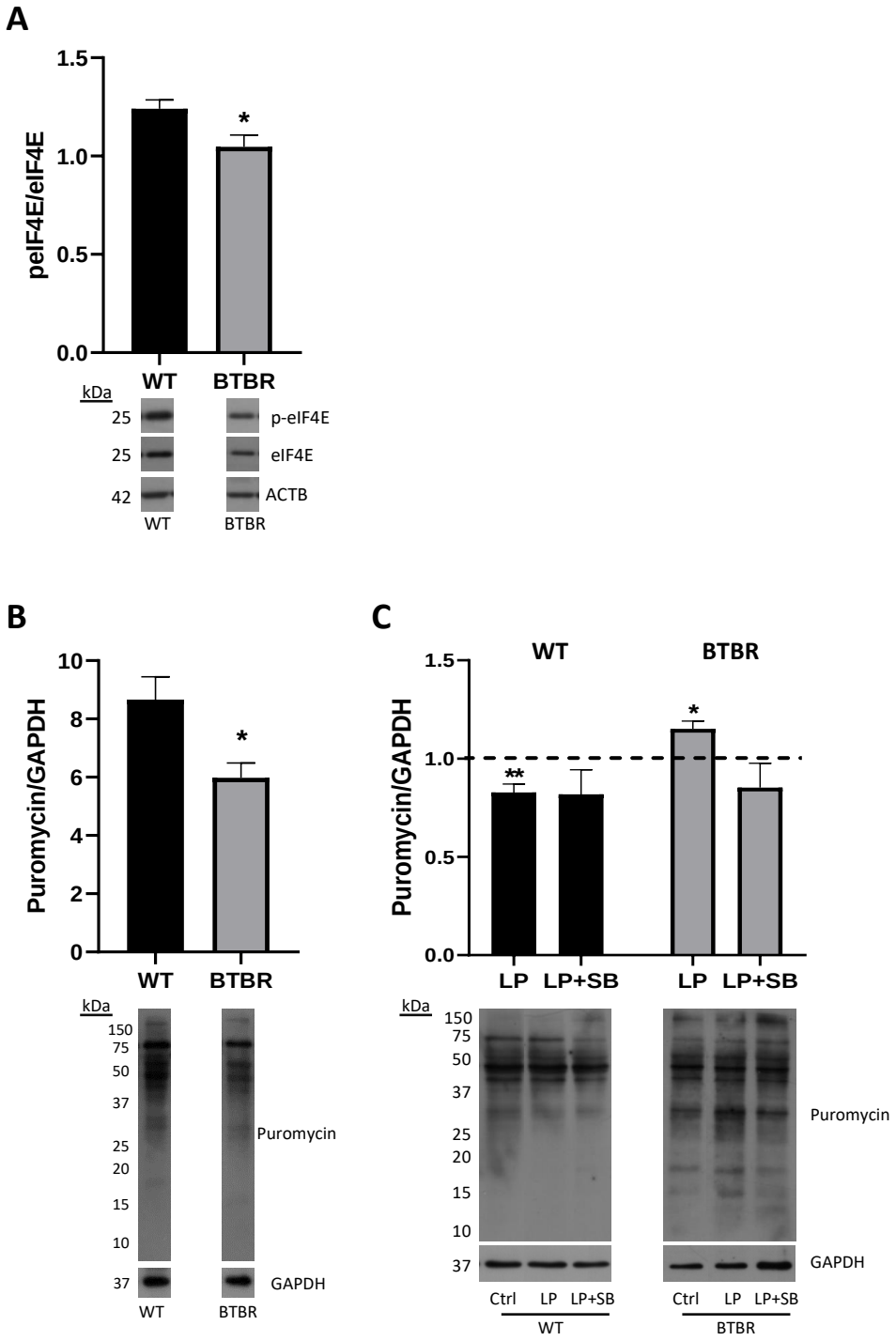
*mean ± SEM. n=5-6. Statistically significant differences are based on unpaired t-test, * p value< 0,05.*

5.2.2 Impairment of synaptic protein synthesis in ASD mouse model: beneficial effect of 5-HT7R stimulation

To investigate the possible impairment of synaptic protein synthesis in BTBR mice, we analyzed the expression levels of the eukaryotic initiation factor 4E (eIF4E), and determined the ratio between its phosphorylated form and its total form in the synaptosomal fraction of BTBR and WT mice. eIF4E is a cap-binding protein involved in the mRNA-ribosome binding step of eukaryotic protein synthesis. The phosphorylation of eIF4E promotes the initiation of translation, thus it can be used as an index of protein synthesis activation (Bramham et al., 2016; Pyronnet, 1999).

Our results showed that the phosphorylation level of eIF4E was significantly lower in synaptosomes from BTBR mice, as compared to WT (Fig. 5A), suggesting an impairment of synaptic protein synthesis in ASD. Accordingly, using SUnSET labelling method on synaptosomes isolated from the brain cortex of BTBR and WT mice, we demonstrated a decreased activity of synaptic protein synthesis in BTBR, as compared to WT (Fig. 5B). Interestingly, 5-HT7R stimulation decreased the level of newly synthetized proteins in WT mice synaptosomes, while it increased local protein synthesis in BTBR mice synaptosomes, rescuing the deficit observed in the pathology (Fig. 5C). The beneficial effect of LP-211 was completely reversed when the agonist was co-

incubated with the 5-HT7R antagonist, SB-269970 (Fig. 5C), confirming the specific involvement of 5-HT7R in rescuing the impairment of synaptic protein synthesis in BTBR mice.


A
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LP+SB
Ctrl
LP
LP+SB
WT
BTBR

Figure 5: Protein synthesis in synaptosomes from cerebral cortex of BTBR and WT mice: effect of 5-HT7R stimulation. (A) *Protein levels of the phosphorylated form of the eukaryotic Initiation Factor 4E (p-eIF4E) normalized to eIF4E in the synaptosomes from WT and BTBR mice brain cortex. β-actin (ACTB) was used as loading control.* ***(B)*** *Levels of puromycinilated proteins normalized on GAPDH in the synaptosomes from BTBR and WT mice brain cortex.* ***(C)*** *Levels of puromycinilated proteins normalized to GAPDH in the synaptosomes from WT and BTBR mice brain cortex incubated with LP-211 (LP, 100 nM) alone, or co-incubated with SB-269970 (SB, 100 nM). Representative images of western blot analysis below the graphs. Molecular weight of each protein, in kDa, is indicated on the left. Data are presented as mean ± SEM. n=5-6. LP and SB values have been compared with the corresponding control value (dotted line). Statistically significant differences are based on unpaired t-test, * p value< 0,05, ** p value<0,01.*

5.3 Angelman syndrome (AS) and alteration of synaptic plasticity

5.3.1 5-HT7R expression levels in hippocampal primary cultures and brain cortex synaptosomes from AS and WT mice

To verify if the stimulation of 5-HT7R also provided beneficial effects in other neurodevelopmental diseases, we used a mouse model of Angelman syndrome (AS). We assessed the expression levels of 5-HT7R in hippocampal neurons from P1-P3 AS and WT mice. No difference was observed between AS and WT animals, indicating that 5-HT7R expression in AS hippocampal neurons is not impaired during neuronal maturation (Fig. 6A).

To extend these analyses to synaptic areas, we investigated 5-HT7R expression levels in brain cortex homogenates from 3 months-old AS and WT mice and in the corresponding synaptosomal fractions. Interestingly, increased expression

levels of 5-HT7R were observed in homogenates of AS mice as compared to WT (Fig. 6B), while reduced 5-HT7R expression levels were detected in AS synaptosomal fractions, as compared to WT (Fig. 6C). These data suggest a selective impairment of 5-HT7R expression in the synaptic areas of AS adult mice brain.

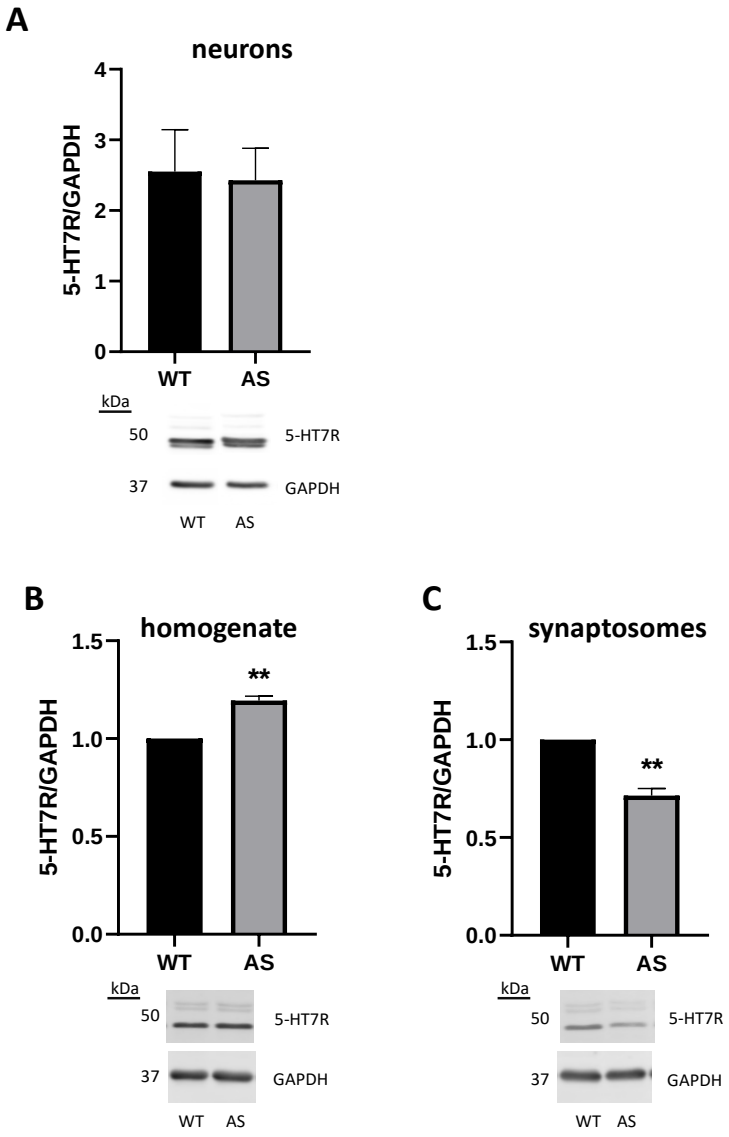


Figure 6: 5-HT7R protein levels in AS and WT hippocampal primary neurons and in synaptosomes from cerebral cortex of AS and WT mice. (A) *5-HT7R protein levels normalized to GAPDH in DIV21 neuronal hippocampal cell cultures from WT and AS mice. n=3. 5-HT7R protein levels normalized to GAPDH in* ***(B)*** *the homogenate and* ***(C)*** *synaptosomes from WT and AS mice cerebral cortex. Representative images of western blot analysis below the graphs. Molecular weight of each protein, in kDa, is indicated on the left. Data are presented as mean ± SEM. n=7-8. Statistically significant differences are based on unpaired t-test, ** p value<0,01.*

5.3.2 Stimulation of 5-HT7R increases protein synthesis in hippocampal neurons from AS and WT mice

To verify the possible alterations of neuronal protein synthesis in AS, we used the SUnSET labelling method to compare the activity of protein synthesis in hippocampal neurons from P1-P3 AS and WT mouse brain. Our data demonstrated that protein synthesis levels in AS hippocampal neurons were comparable to those in WT (Fig. 7A), indicating that the neuronal machinery of protein synthesis is not impaired in AS. Interestingly, acute stimulation of hippocampal neurons with LP-211 increased levels of newly synthetized proteins in both AS and WT (Fig. 7B), and this increase was prevented by coincubation of LP-211 with the receptor antagonist SB-269970 (Fig. 7B).

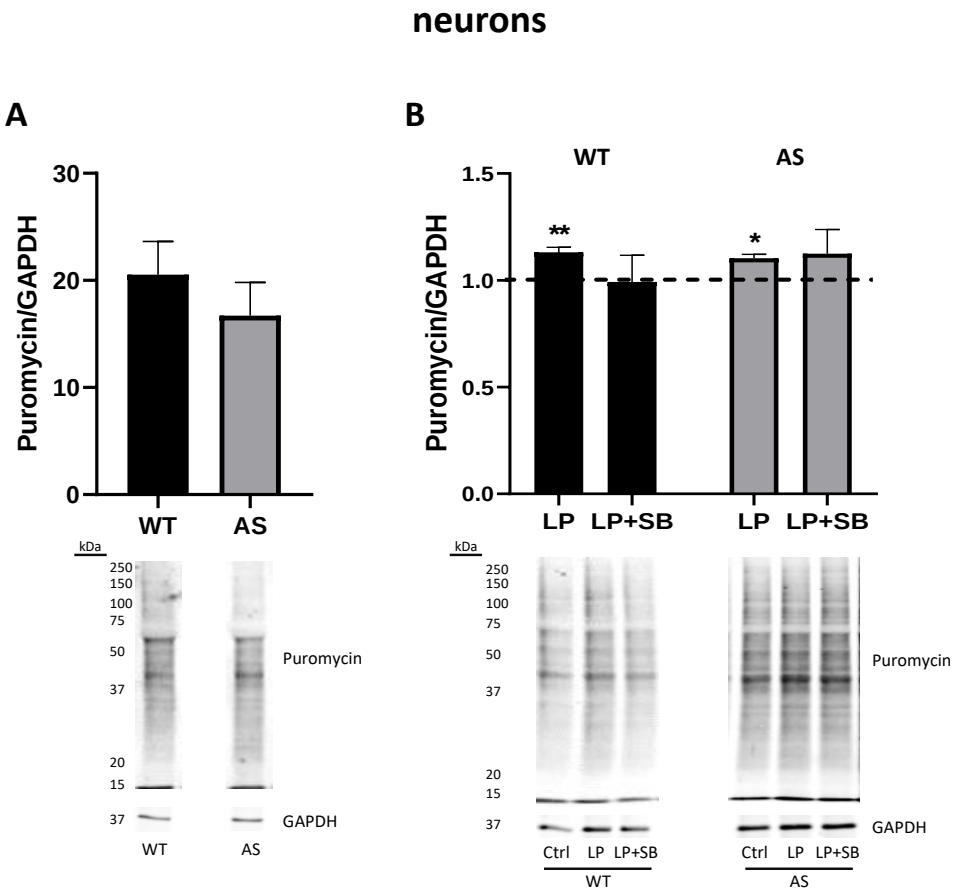


Figure 7: Protein synthesis in hippocampal neurons from AS and WT mice: effect of 5-HT7R stimulation. (A) *Levels of puromycinilated proteins normalized to GAPDH in DIV7 neuronal hippocampal cell cultures from WT and AS mice.* ***(B)*** *Levels of puromycinilated proteins normalized to GAPDH in DIV7 neuronal hippocampal cell cultures from WT and AS mice incubated with LP-211 (LP, 100 nM) alone, or co-incubated with SB-269970 (SB, 100 nM). Representative images of western blot analysis below the graphs. Molecular weight of each protein, in kDa, is indicated on the left. Data are presented as mean ± SEM. n=3. LP and SB values have been compared with the corresponding control value (dotted line). Statistically significant differences are based on unpaired t-test, * p<0,05, ** p value<0,01.*

5.3.3 Impairment of synaptic protein synthesis in AS mouse model: beneficial effects of 5-HT7R stimulation

To investigate if the synaptic system of protein synthesis was affected in AS mice, we studied the levels of protein synthesis in synaptosomes from AS and WT mouse brain, using the SUnSET labelling method. We observed a significant reduction in synaptic protein synthesis in AS mice, as compared to WT (Fig. 8A), suggesting that synaptic protein synthesis is selectively altered in AS. Interestingly, stimulation of synaptosomes with LP-211 led to a decrease of newly synthetized proteins in WT, but to a significant increase in AS (Fig. 8B). Coincubation with SB-269970 prevented the LP-211-dependent increase of protein synthesis in AS mice, but did not reverse the effect of LP-211 in WT mice (Fig. 8B). Altogether, these data suggest that the alterations of the synaptic machinery of protein synthesis in AS is reversed by stimulation of 5-HT7R, while its activation in a non-pathological model leads to impairment of the synaptic protein synthesis, which does not seem to depend exclusively on 5-HT7R.

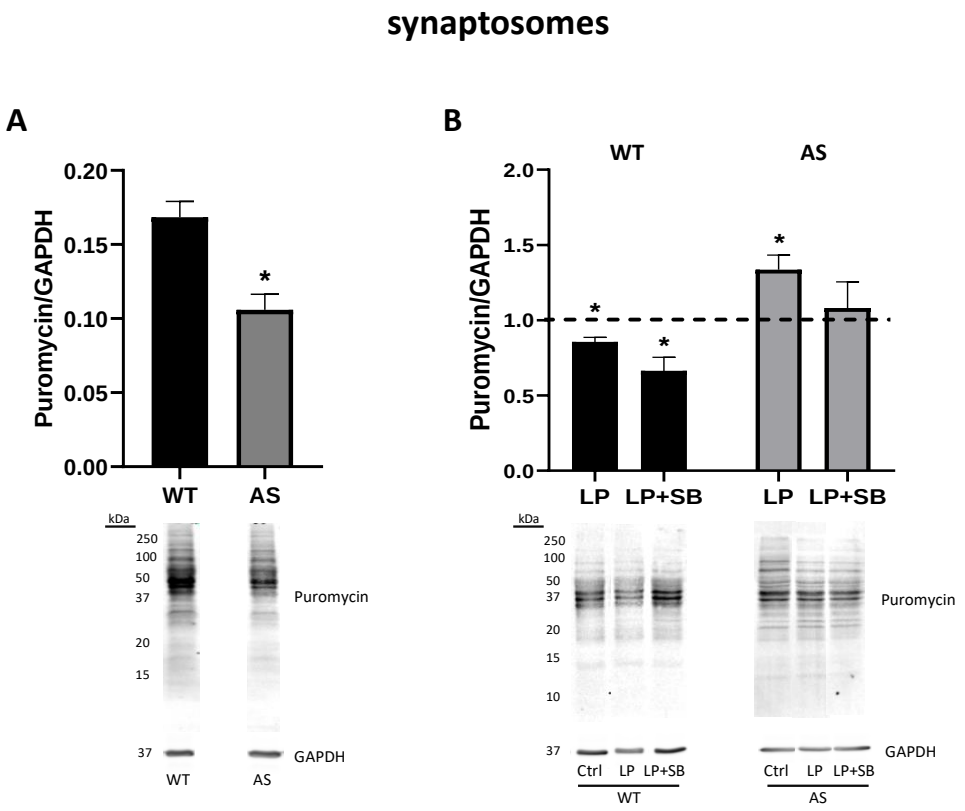


Figure 8: Protein synthesis in synaptosomes from AS and WT mice cerebral cortex: effect of 5-HT7R stimulation. (A) *Levels of puromycinilated proteins normalized to GAPDH in the synaptosomes from WT and AS mice brain cortex.* ***(B)*** *Levels of puromycinilated proteins normalized to GAPDH in the synaptosomes from WT and AS mice brain cortex incubated with LP-211 (LP, 100 nM) alone, or co-incubated with SB-269970 (SB, 100 nM). Representative images of western blot analysis below the graphs. Molecular weight of each protein, in kDa, is indicated on the left. Data are presented as mean ± SEM. n=5-7. LP and SB values have been compared with the corresponding control value (dotted line). Statistically significant differences are based on unpaired t-test, * p<0,05.*

5.3.4 Alterations of dendritic spine density in AS: rescuing effects of 5-HT7R stimulation

A decrease in dendritic spines density was previously reported in AS neurons (Baudry et al., 2012). Since stimulation of 5-HT7R with LP-211 is able to enhance spinogenesis (Speranza et al., 2017), we hypothesized that LP-211 treatment could rescue alterations of spines density in AS neurons. We isolated hippocampal neurons from P1-P3 AS and WT mice and performed a chronic stimulation (72 h) with LP-211, SB-269970, or a combination of both. As expected, we observed a significant decrease in dendritic spines density in AS neurons, as compared to WT (Fig. 9).

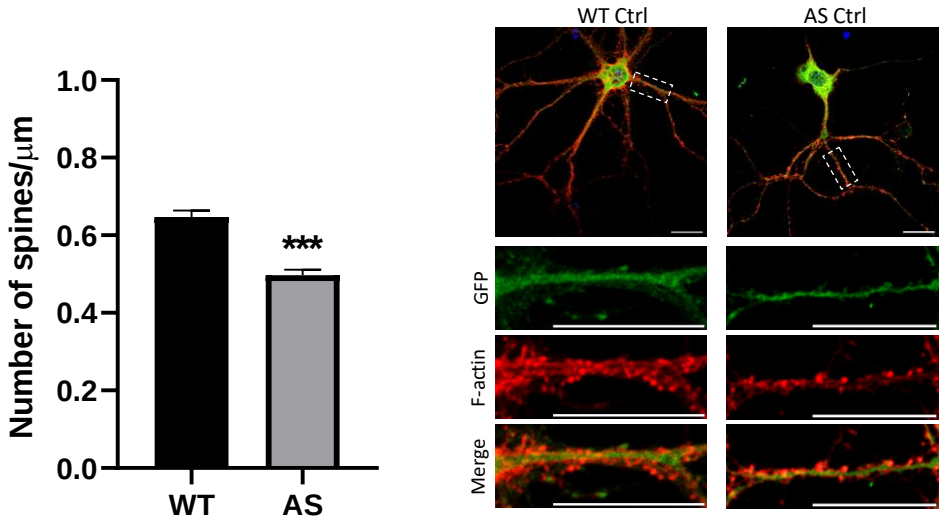


Figure 9: Impairment of dendritic spine density in AS hippocampal neurons. *Dendritic spine density of WT and AS hippocampal neurons at DIV21, infected with GFP lentivirus and immunostained with F-actin. n=50-53 neurons from 3 independent experiments were analysed for each group. Representative*

*images on the right. Statistically significant differences are based on unpaired t-test, *** p<0.001. Scale bar: 20 µm.*

Activation of 5-HT7R with LP-211 increased dendritic spine density in neurons from both WT and AS mice (Fig. 10,11). Coincubation of neurons with LP-211 and SB-269970 abolished the 5-HT7R activation effects on dendritic spines in WT, reducing the density of dendritic spines to a value lower than control (Fig. 10).

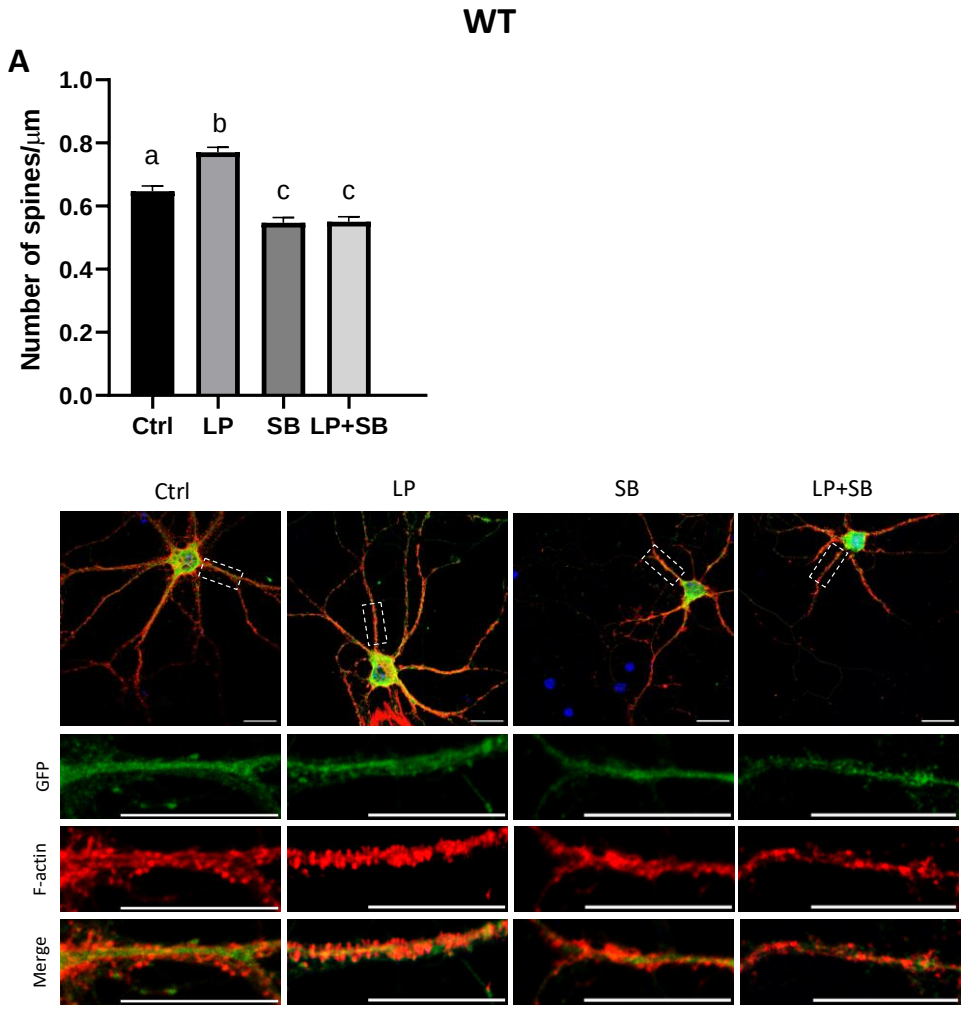


Figure 10: Effect of 5-HT7R stimulation on dendritic spine density of WT hippocampal neurons. *Dendritic spine density of WT hippocampal neurons at DIV21, infected with GFP lentivirus, incubated with LP-211 (LP, 100nm), SB-269970 (SB, 100nm) or both, and immunostained with F-actin. n=50-53 neurons from 3 independent experiments were analysed for each group. Representative images on the right. Different letters on top of each histogram indicate statistically significant differences by one-way ANOVA with Tukey's post-test, p<0.001. Ctrl: control. Scale bar 20 µm.*

In contrast, in AS mice, coincubation of neurons with LP-211 and SB-269970 significantly reduced dendritic spine density, as compared to LP-211 alone, but the value remained significantly higher than in the control (Fig. 11). SB-269970 alone significantly decreased dendritic spine density in WT neurons but increased it in AS neurons, as compared to control (Fig. 10,11).

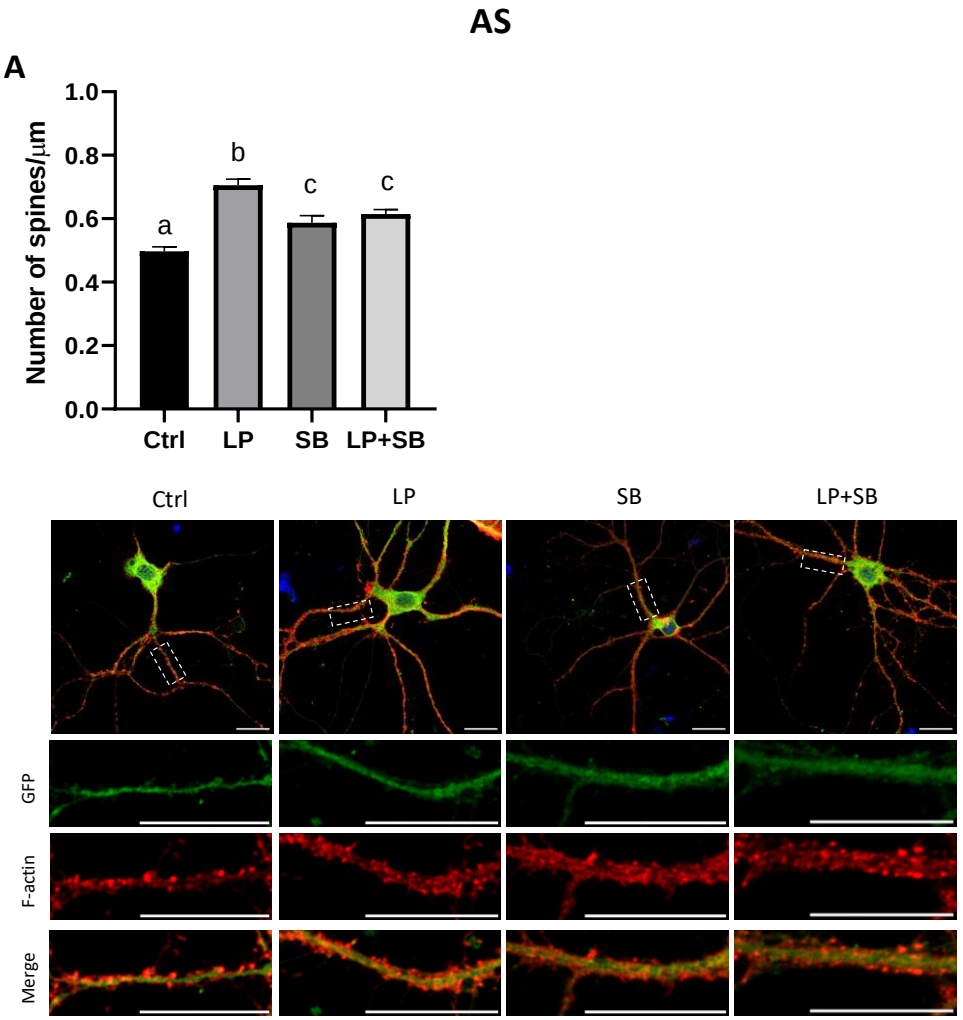


Figure 11: Effect of 5-HT7R stimulation on dendritic spine density of AS hippocampal neurons. *Dendritic spine density of AS hippocampal neurons at DIV21, infected with GFP lentivirus, incubated with LP-211 (LP, 100nm), SB-269970 (SB, 100nm) or both, and immunostained with F-actin. n=38-50 neurons from 3 independent experiments were analysed for each group. Representative images on the right. Different letters on top of each histogram indicate statistically significant differences by one-way ANOVA with Tukey's post-test, p<0.001. Ctrl: control. Scale bar 20 µm.*

It is noteworthy that stimulation of 5-HT7R in AS neurons increased dendritic spine density not only compared to AS with vehicle treatment, but also compared to WT (Fig. 12). These data suggest that activation of 5-HT7R is able to ameliorate morphological deficits related to AS pathology.

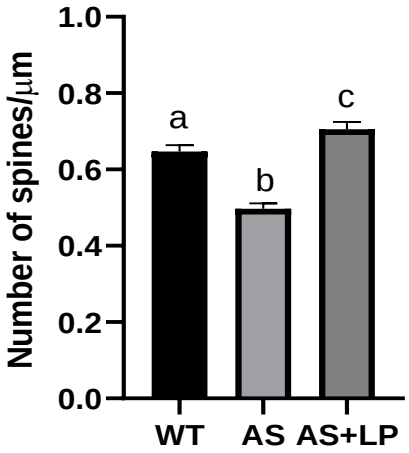


Figure 12: 5-HT7R stimulation rescues the dendritic spine density impairment of AS hippocampal neuron. *Dendritic spine density of WT and AS hippocampal neurons at DIV21, incubated with or without LP-211 (LP, 100nm), infected with GFP lentivirus, and immunostained with F-actin. n=45-53 neurons from 3 independent experiments were analysed for each group. Different letters on top of each histogram indicate statistically significant differences by one-way ANOVA with Tukey's post-test, p<0.001.*

5.4 Alterations of synaptic plasticity in Progressive Myoclonic Epilepsy type 1 (EPM1)

5.4.1 Alteration of neuronal morphology in human EPM1 neurons

To assess possible morphological changes in EPM1 neurons, we differentiated neurons from human NPCs derived from iPSCs of EPM1 patients in order to obtain a human neuronal model of the disease. We used Neurolucida imaging system to analyze GFP-sparsely-labeled 8 weeks-old neurons, and to perform their 3D morphological reconstruction (Fig. 13). Interestingly, while no significant morphological changes were detected in the cell body of EPM1 neurons (Fig. 13A,B), the neurites of these neurons were strikingly different in shape and number from the controls (Fig. 13C,D,E). In particular, EPM1 neurons exhibited longer (total length, Fig. 13C) and more branched neurites (nodes, Fig. 13E), as compared to controls, suggesting a more complex neuronal morphology.

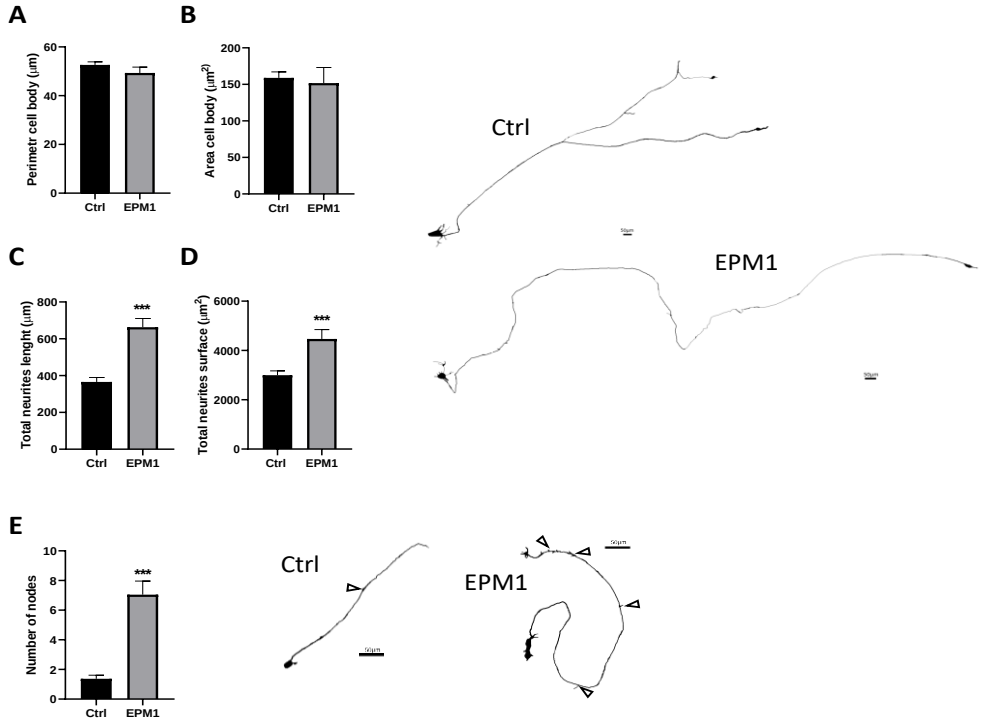


Figure 13: Morphological analysis of control and EPM1 human neurons. *Quantification of the cell body* ***(A)*** *perimeter and* ***(B)*** *area, and neurites* ***(C)*** *total length,* ***(D)*** *total surface and* ***(E)*** *number of nodes of control (Ctrl) and EPM1 8-weeks human neurons, reconstructed using Neurolucida software. Representative images on the right. Scale bar 50 µm. Arrows indicate nodes. Data are presented as mean ± SEM. n=53 Ctrl and n=48 EPM1 neurons. Statistically significant differences are based on nonparametric test Mann Whitney, *** p value< 0,001.*

The increased neuronal complexity of EPM1 neurons was confirmed by Sholl analysis, which indicated a higher number of intersections and nodes in these neurons, especially in the first 200 µm away from the soma (Fig. 14A,B). Interestingly, although EPM1 neurites were longer and more branched than controls, they were significantly thinner (Fig. 14C).

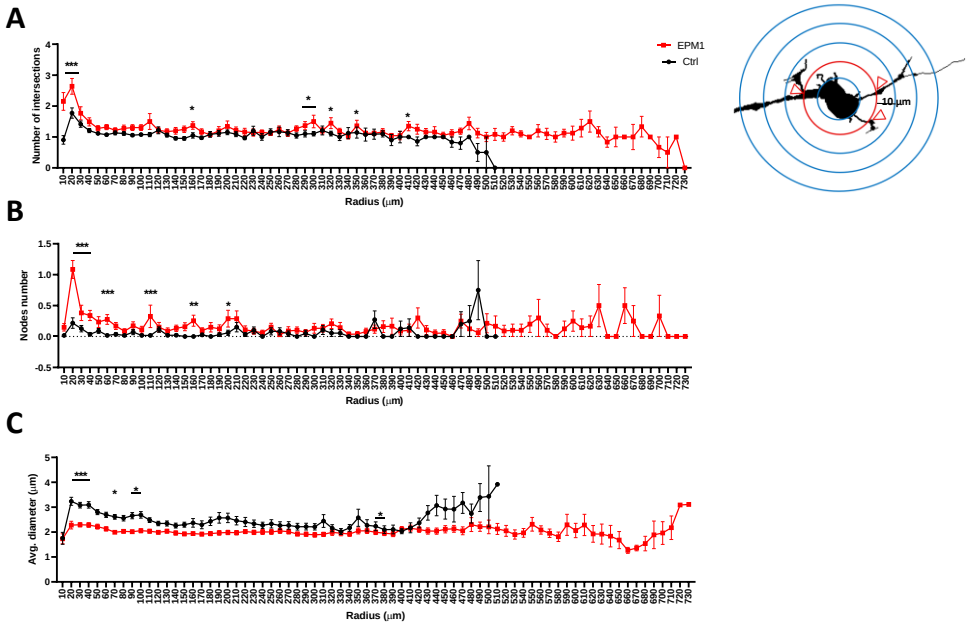


Figure 14: Sholl analysis of control and EPM1 human neurons. *Number of* ***(A)*** *intersection,* ***(B)*** *nodes, and* ***(C)*** *average diameter of control (Ctrl) and EPM1 8-weeks human neurons, reconstructed using Neurolucida software. Scheme of concentric circles, with a radius increment of 10 µm, used for the Sholl analysis on the right. Data are presented as mean ± SEM. n=53 Ctrl and n=48 EPM1 neurons. Statistically significant differences are based on two-way ANOVA followed by Sidak's multiple comparisons test, * p value< 0,05, ** p value< 0,01, *** p value< 0,001.*

5.4.2 EPM1 human cerebral organoids (hCOs) display alterations in the synaptic area

We isolated synaptosomes from hCOs, obtained from EPM1 patients and controls, at different maturational stages. As expected, CSTB level was

significantly reduced in protein extracts of EPM1 hCOs (Fig. 15A), as well as in the corresponding synaptosomal fractions at all stages analyzed (Fig. 15B). A key role of CSTB in synaptic plasticity was previously indicated by its local synthesis and secretion from synaptic terminals (Penna et al., 2019), and by its involvement in vesicle trafficking (Joensuu et al., 2014). Therefore, we hypothesized that the low levels of CSTB observed in synaptic areas of EPM1 hCOs may affect synaptic functions and EVs release.

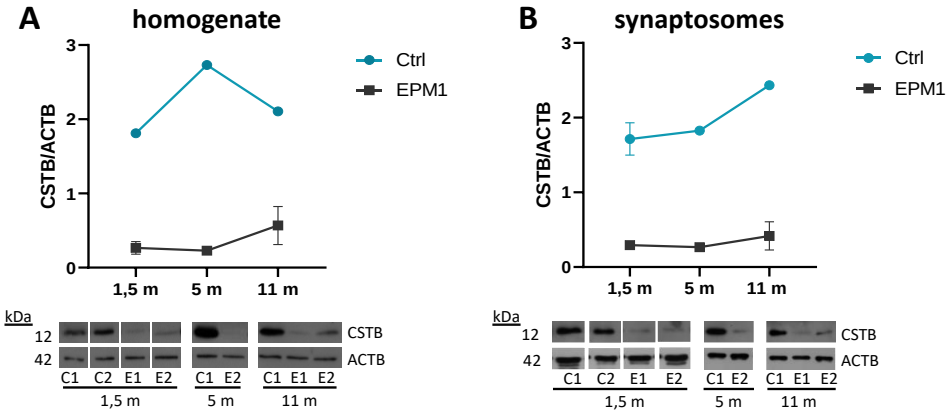


Figure 15: CSTB protein levels in homogenate and synaptosomes from EPM1 human cerebral organoids. *Cystatin B (CSTB) protein levels normalized to β-actin (ACTB) in* ***(A)*** *homogenate and* ***(B)*** *synaptosomes from EPM1 and control (Ctrl) hCOs. Representative images of western blot analysis below the graphs. Molecular weight of each protein, in kDa, is indicated on the left. Data are presented as mean ± SD. n=1-2, with each time point as a pool of 20-40 hCOs. Controls: C1, C2; EPM1 patients: E1, E2; m: months.*

To investigate alterations of the synaptic area in the hCOs of EPM1 patients, we evaluated the synaptosomal expression levels of three synaptic hallmarks

at different developmental stages. Interestingly, synaptophysin was reduced in EPM1 synaptosomes at all maturational stages analyzed (Fig. 16A), suggesting an impairment of synaptic vesicles in the pathology. In contrast, levels of the other two presynaptic markers, syntaxin (Fig. 16B) and synaptotagmin 1/2 (Fig. 16C), were reduced only in the early stages of maturation, while their expression levels became comparable to the control hCOs at later maturation stages (Fig, 16B,C).

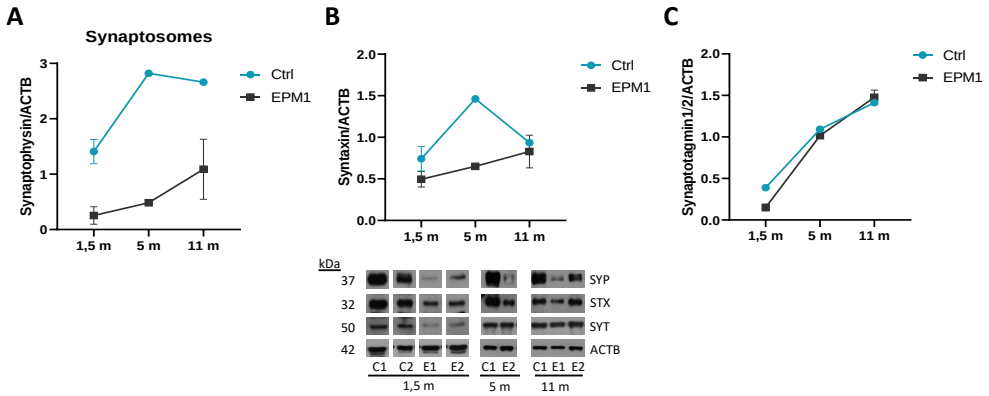


Figure 16: Synaptic proteins levels in synaptosomes from EPM1 human cerebral organoids. *Protein levels of* ***(A)*** *synaptophysin (SYP),* ***(B)*** *syntaxin (STX) and* ***(C)*** *synaptotagmin 1/2 (SYT) normalized to β-actin (ACTB) in synaptosomes from EPM1 and control (Ctrl) hCOs. Representative images of western blot analysis below the graphs. Molecular weight of each protein, in kDa, is indicated on the left. Data are presented as mean ± SD. n=1-2, with each time point as a pool of 20-40 hCOs. Controls: C1, C2; EPM1 patients: E1, E2; m: months.*

To extend our investigation on altered synaptic functions in EPM1, we studied synaptosomal expression levels of the eukaryotic initiation factor eIF4G2,

which is axonally synthesized, as eIF2B2 (Kar et al., 2013). We observed a decrease in eIF4G2 levels in EPM1 synaptosomes, as compared to controls (Fig. 17B). It is noteworthy that this decrease is specifically related to the synaptic area and is not generalized to the corresponding total protein extract (Fig. 17A). These results suggest that depletion of CSTB in synaptic area is linked to an impairment of synaptic protein synthesis, which has been demonstrated to be crucial for the nerve terminal plasticity (Perrone-Capano et al., 2021).

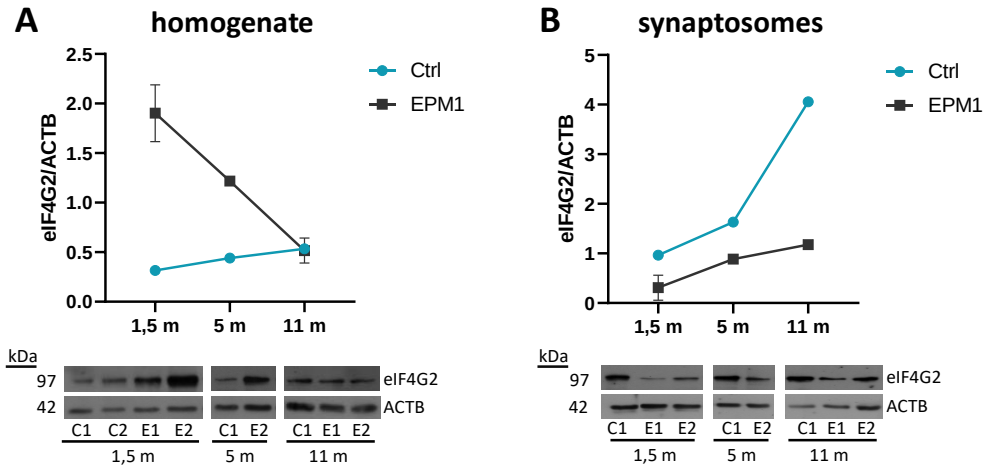


Figure 17: eIF4G2 protein levels in homogenate and synaptosomes from EPM1 human cerebral organoids. *Protein levels of the eukaryotic Initiation Factor 4G2 (eIF4G2) normalized to β-actin (ACTB) in* ***(A)*** *homogenates and* ***(B)*** *synaptosomes from EPM1 and control (Ctrl) hCOs. Representative images of western blot analysis below the graphs. Molecular weight of each protein, in kDa, is indicated on the left. Data are presented as mean ± SD. n=1-2, with each time point as a pool of 20-40 hCOs. Controls: C1, C2; EPM1 patients: E1, E2; m: months.*

Interestingly, in synaptosomes from EPM1 patients we also observed a significant reduction of the expression levels of EVs markers such as CD9 and CD81 (Fig. 18A,B), which further supports the idea that the deficiency in CSTB is linked to dysfunctions of synaptic terminals, and that the impairment of the synaptic secretion machinery also contributes to the pathology.

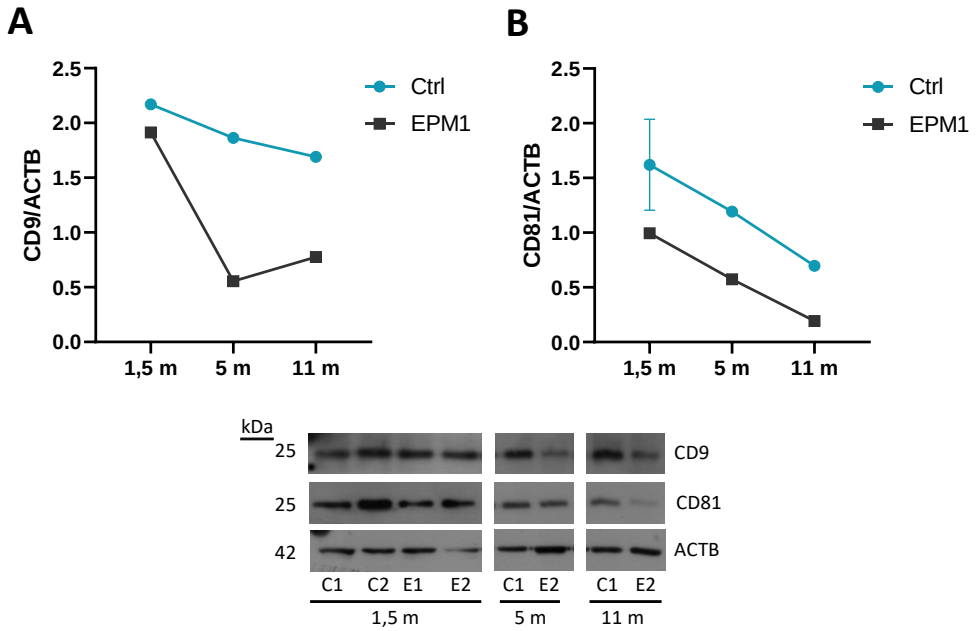


Figure 18: EVs markers protein levels in synaptosomes from EPM1 human cerebral organoids. *Protein levels of* ***(A)*** *CD9 and* ***(B)*** *CD81 normalized to β-actin (ACTB) in the synaptosomes from EPM1 and control (Ctrl) hCOs. Representative images of western blot analysis below the graphs. Molecular weight of each protein, in kDa, is indicated on the left. Data are presented as mean ± SD. n=1-2, with each time point as a pool of 20-40 hCOs. Controls: C1, C2; EPM1 patients: E1, E2; m: months.*

To corroborate this hypothesis, we performed mass spectrometric analysis of proteins from homogenates (h) and synaptosomal (syn) fractions of control

and patients hCOs. A set of 76 proteins was found to be enriched in synaptosomal fractions of control hCOs, as compared to homogenates (ratio syn/h ≥ 1,2) (Fig. 19A). We then investigated their abundance in synaptosomes of hCOs derived from EPM1 patients. Intriguingly, none of these 76 proteins were enriched in the synaptosomes derived from EPM1 patients. Instead, 31 of them were expressed at lower levels in patient synaptosomes, as compared to the total protein extract (ratio syn/h ≤ 0,8) (Fig. 19A). Gene ontology analysis performed using the String database (string-db.org) on these 31 proteins indicated that they are involved in pathways related to secretion and vesicle-mediated transport (Fig. 19B). In EPM1 hCOs, only 9 proteins were enriched in the synaptosomal fraction (ratio syn/h ≥ 1,2), and all of them were expressed at lower levels in the synaptosomal fraction from control hCOs (ratio syn/h ≤ 0,8), supporting the idea that the protein composition of the synaptic area of EPM1 hCOs is profoundly altered. It is noteworthy that among these 9 proteins, we detected two proteins, ERGIC and GBF1, which are involved in vesicular trafficking and secretion.

Altogether, these results strongly suggest that the deficiency of CSTB contributes to deregulation of synaptic functions, altering EVs trafficking and release at the presynaptic bouton.

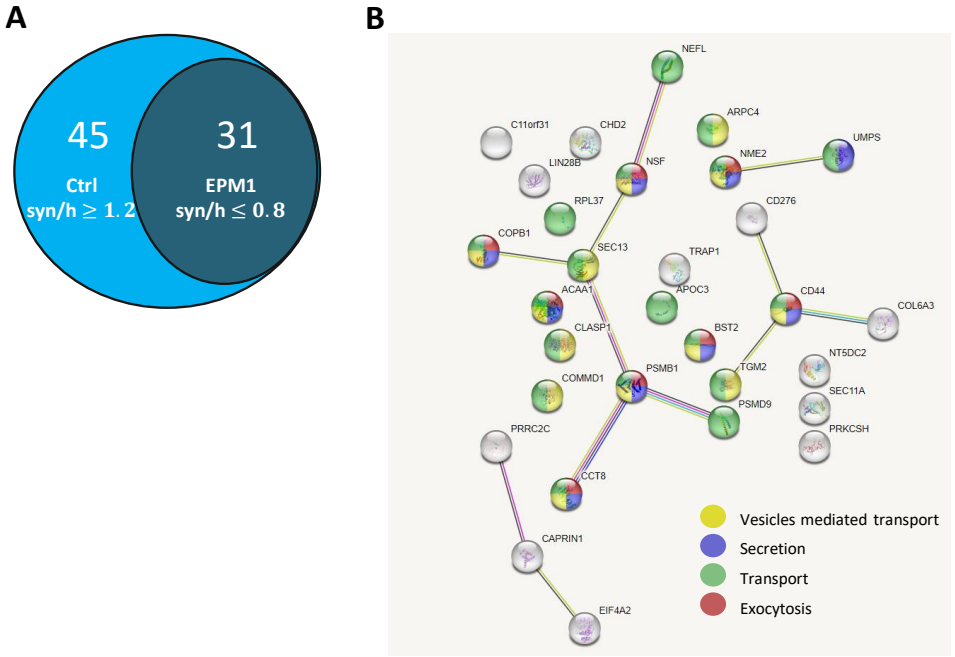


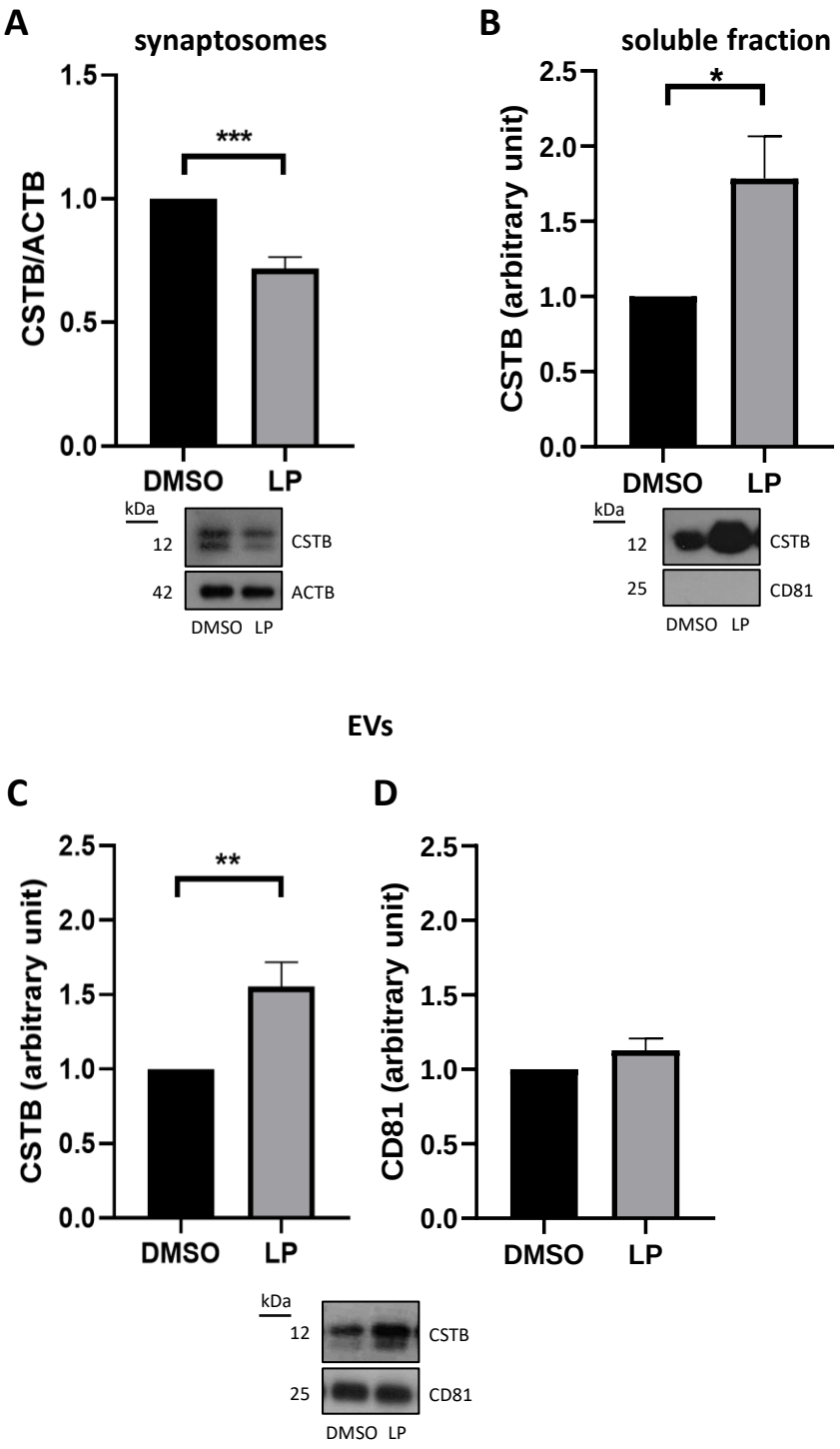
Figure 19: Mass spectrometry analysis of homogenate and synaptosomes from control and EPM1 human cerebral organoids. (A) *Venn diagram of the proteins identified by Mass Spectrometry as enriched in the synaptosomes (syn) versus homogenate (h) from 1,5 months control hCOs (Ctrl) (76 proteins, syn/h ≥1,2), and depleted in synaptosomes versus homogenate of 1,5 months EPM1 HCOs (31 proteins, syn/h ≤0,8).* ***(B)*** *Gene ontology analysis of the 31 proteins depleted in EPM1 synaptosomes using String database.*

5.5 Secretion of CSTB from brain synaptosomes

5.5.1 CSTB secretion from rat brain cortex synaptosomes: modulation by 5-HT7R stimulation

It has been previously demonstrated that CSTB is locally synthetized in nerve endings (Penna et al., 2019). Given the effect of 5-HT7R stimulation on synaptic protein synthesis (Fig. 3), we evaluated expression levels of CSTB in rat synaptosomes incubated with LP-211. Our results indicated a significant decrease in CSTB after incubation of synaptosomes with the 5-HT7R agonist, LP-211 (Fig. 20A), supporting our results indicating a reduction in synaptic protein synthesis following 5-HT7R stimulation. It is noteworthy that CSTB migrates as a double band, as was previously reported by other groups (Alakurtti et al., 2005). The explanation of this is not clear and requires further analysis, although it is possible to hypothesize that the double band depends on post-translational modification.

Based on our previous demonstration that CSTB is released by brain synaptosomes (Penna et al., 2019), we determined whether CSTB release from rat synaptosomes could be affected by 5-HT7R stimulation. Our data indicated that CSTB was secreted both as a soluble protein and associated with the EV fraction, and that both secretion modalities were enhanced by 5-HT7R stimulation (Fig. 20B,C). On the other hand, the expression levels of the exosomal marker CD81 did not change in the EVs fraction after stimulation of 5-HT7R (Fig. 20D). These results indicate that 5-HT7R activation selectivity affects the release of CSTB from synaptosomes.


A
synaptosomes
CSTB/ACTB
1.5
1.0
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***
DMSO
LP
kDa
12
CSTB
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ACTB
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B
soluble fraction
CSTB (arbitrary unit)
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12
CSTB
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CD81
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EVs
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CSTB (arbitrary unit)
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1.5
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0.0
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LP
kDa
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CSTB
25
CD81
DMSO LP

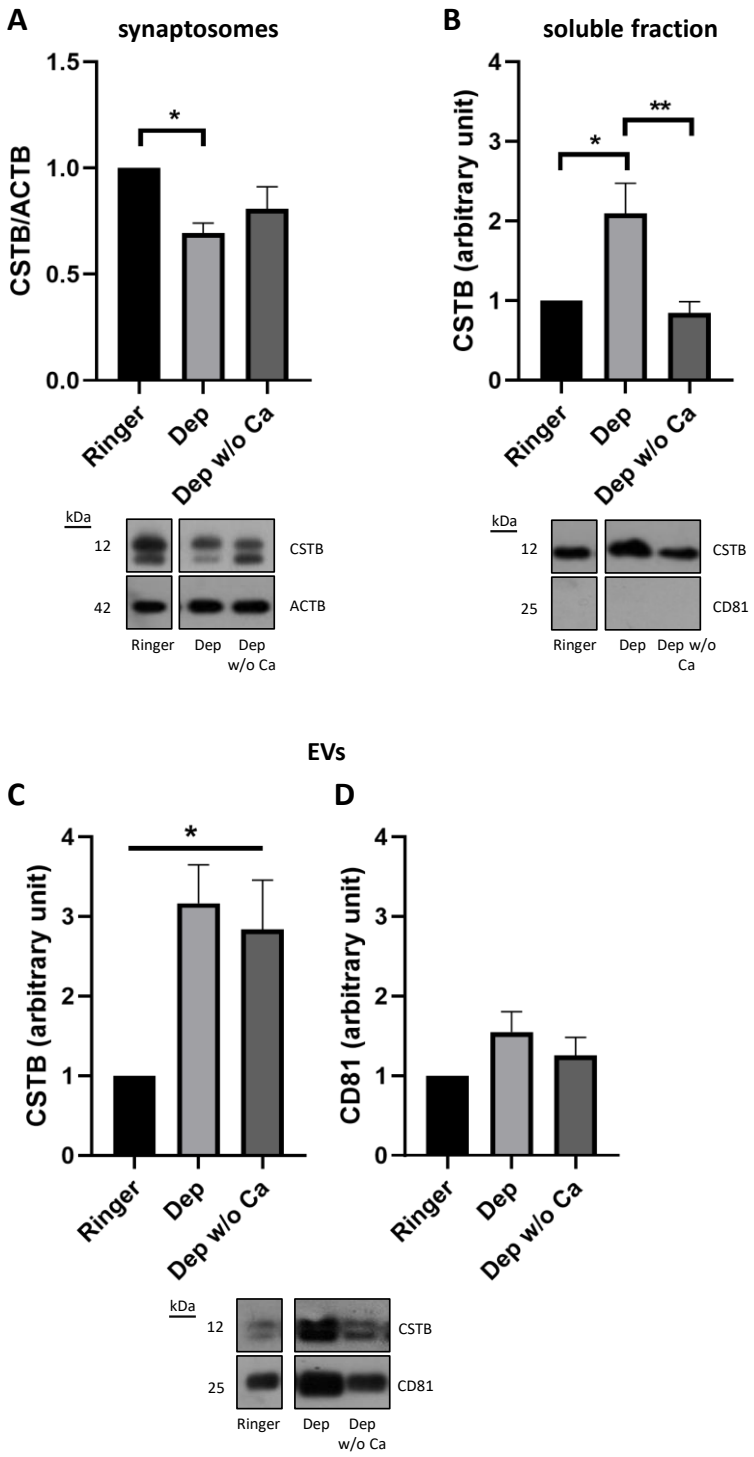
Figure 20: 5-HT7R stimulation modulates CSTB protein levels and release from rat brain cortex synaptosomes. (A) *Cystatin B (CSTB) protein levels normalized to β-actin (ACTB) in rat brain cortex synaptosomes incubated with LP-211 (LP, 100 nM) or its vehicle (DMSO).* ***(B)*** *CSTB protein levels in the soluble fraction secreted from rat brain cortex synaptosomes incubated with LP or DMSO. CSTB* ***(C)*** *and CD81* ***(D)*** *protein levels in EVs fraction secreted from rat brain cortex synaptosomes incubated with LP or DMSO. Representative images of western blot analysis below the graphs. Molecular weight of each protein, in kDa, is indicated on the left. Data are presented as mean ± SEM. n=3-5. Statistically significant differences are based on unpaired t-test * p value<0.05, ** p value< 0,01, *** p value< 0,001.*

5.5.2 CSTB secretion from rat brain cortex synaptosomes: modulation by calcium ions

We have previously demonstrated that CSTB release from synapses is influenced by depolarization (Penna et al., 2019). To investigate the release mechanisms of this protein, we studied the role of calcium ions in the depolarization-dependent CSTB release from rat brain cortex synaptosomes.

CSTB expression levels decreased significantly in rat brain cortex synaptosomes incubated with a depolarizing medium, as compared to a physiological medium (Ringer) (Fig. 21A), while CSTB levels did not change when synaptosomes were incubated with a depolarizing medium deprived of calcium ions (Fig. 21A). These data suggest that the effect of depolarizing incubation medium on synaptic CSTB expression level is calcium-dependent. Interestingly, CSTB release from synaptosomes, in a soluble form and associated to EV fraction, is increased by depolarization (Fig. 21B,C). When

the depolarizing medium was deprived of calcium ions, CSTB release as a soluble protein returned to the control value, indicating that this depolarization-dependent increased release is mediated by calcium ions (Fig. 21B). On the other hand, in presence of depolarizing medium deprived of calcium ions the release of CSTB *via* EVs was still significantly higher than in control (Fig. 21C), suggesting different regulation mechanisms of CSTB release from synaptosomes as a soluble protein or associated to EVs. Interestingly, the expression pattern of the exosomal marker CD81 in the EVs fraction, in depolarizing condition with or without calcium, did not change, as compared to control (Fig. 21D).


A
synaptosomes
CSTB/ACTB
1.5
1.0
0.5
0.0
*
Ringer
Dep
Dep w/o Ca
kDa
12
42
CSTB
ACTB
Ringer
Dep
Dep w/o Ca
B
soluble fraction
CSTB (arbitrary unit)
4
3
2
1
0
*
**
Ringer
Dep
Dep w/o Ca
kDa
12
25
CSTB
CD81
Ringer
Dep
Dep w/o Ca
EVs
C
CSTB (arbitrary unit)
4
3
2
1
0
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Ringer
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Dep w/o Ca
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12
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CSTB
CD81
Ringer
Dep
Dep w/o Ca
D
CD81 (arbitrary unit)
4
3
2
1
0
Ringer
Dep
Dep w/o Ca

Figure 21: The depolarization-dependent CSTB secretion from rat brain cortex synaptosomes depends on extracellular calcium levels. (A) *Cystatin B (CSTB) protein levels normalized to β-actin (ACTB) in rat brain cortex synaptosomes incubated in ringer medium, depolarizing medium (Dep) or depolarizing medium deprived of calcium ions (Dep w/o Ca).* ***(B)*** *CSTB protein levels in the soluble fraction secreted from rat brain cortex synaptosomes incubated in ringer medium, Dep or Dep w/o Ca.* ***(C)*** *CSTB and* ***(D)*** *CD81 protein levels in EVs fraction secreted from rat brain cortex synaptosomes incubated in ringer medium, Dep or Dep w/o Ca. Representative images of western blot analysis below the graphs. Molecular weight of each protein, in kDa, is indicated on the left. Data are presented as mean ± SEM. n=3-5. Statistically significant differences are based on one-way ANOVA with Tukey's post-test, * p value<0.05, ** p value<0,01.*

To further investigate the role of calcium ions in CSTB secretion mechanisms, we modulated intracellular calcium levels by incubating synaptosomes in the presence of BAPTA-AM, a cell-permeant calcium chelator, which decreases intracellular calcium levels, or in the presence of the calcium ionophore ionomycin, which increases intracellular calcium levels. While no differences were observed in synaptosomal CSTB levels with BAPTA-AM, the presence of ionomycin decreased CSTB levels (Fig. 22A). On the other hand, the secretion of CSTB as soluble protein decreased in the presence of BAPTA-AM, as well as in the presence of ionomycin (Fig. 22B). The expression levels of the exosomal marker CD81 in EVs fraction decreased significantly in the presence of BAPTA-AM or ionomycin, thus displaying a similar pattern as CSTB in response to intracellular calcium ion modulation (Fig. 22C,D). These data confirmed that, at least in part, the release of CSTB is associated with CD81-positive EVs.

Altogether, our results indicate that calcium homeostasis is crucial for CSTB synaptic secretion.

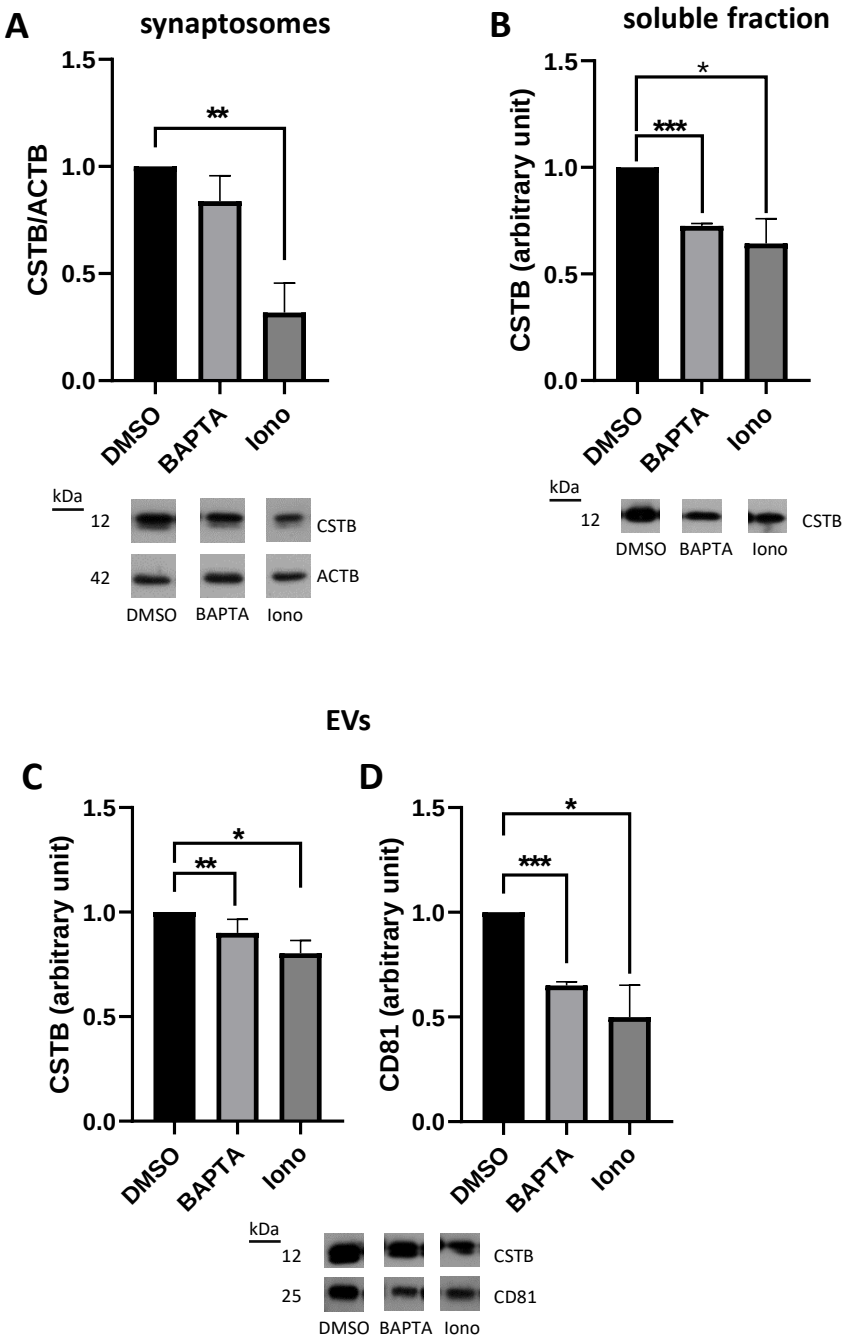

Figure 22: CSTB secretion from rat brain cortex synaptosomes depends on intracellular calcium levels. (A) *Cystatin B (CSTB) protein levels normalized to β-actin (ACTB) in rat brain cortex synaptosomes incubated in presence of BAPTA-AM (BAPTA, 2 µM), ionomycin (iono, 100 nM) or their vehicle (DMSO).* ***(B)*** *CSTB protein levels in the soluble fraction secreted from rat brain cortex synaptosomes incubated in presence of BAPTA, iono, or DMSO.* ***(C)*** *CSTB and* ***(D)*** *CD81 protein levels in EVs fraction secreted from rat brain cortex synaptosomes incubated in presence of BAPTA, iono, or DMSO. Representative images of western blot analysis below the graphs. Molecular weight of each protein, in kDa, is indicated on the left. Data are presented as mean ± SEM. n=3-5. Statistically significant differences are based on unpaired t-test, * p value<0.05, ** p value< 0,01, *** p value< 0,001.*

5.5.3 CSTB secretion from synaptosomes of embryonic mouse cortices and hCOs

To extend our investigation of CSTB synaptic secretion to embryonic stages, we isolated synaptosomes from E18 mouse cortices. We found that 43% of SYP-positive synaptosomes were CSTB-positive (Fig. 23A), and 37% were CD81-positive (Fig. 23B), confirming that, also during development, synaptic regions contain CSTB and at least one EVs marker.

We also analyzed EVs secreted by these synaptosomes and we found that some of the CD81-positive EVs were also CSTB-positive (Fig. 23C). Moreover, we demonstrated that synaptosomes isolated from hCOs were able to release CSTB both as a soluble protein and associated with EVs fraction (Fig. 23D), confirming in the human model that CSTB is secreted from synaptic areas both as soluble protein and *via* EVs.

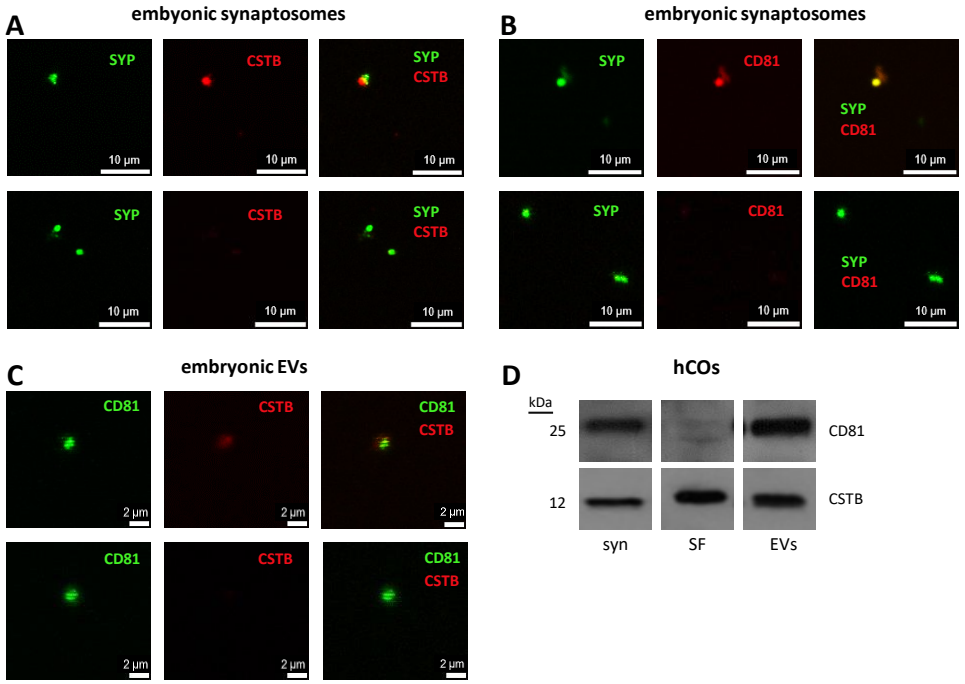

Figure 23: CSTB secretion from synaptosomes of embryonic mouse brain and human cerebral organoids. (A) *Micrographs of synaptosomes from E18 mice brain immunostained with synaptophysin (SYP, green) and Cystatin B antibody (CSTB, red).* ***(B)*** *Micrographs of synaptosomes isolated from E18 mice brain immunostained with SYP (green) and CD81 antibody (red).* ***(C)*** *Micrographs of EVs secreted from E18 mouse brain synaptosomes immunostained with CD81 (green) and CSTB antibody (red). Scale bar in each panel. n=5* ***(D)*** *Representative images of Western blot analysis of synaptosomes (syn), soluble fraction (SF) and EVs fraction from hCOs, using CSTB and CD81 antibodies.*

6. DISCUSSION

Several neurodevelopmental and neurodegenerative diseases are associated with dysfunction of neuronal activity due to perturbations at the synaptic level (Torres et al., 2017). For instance, Alzheimer's, Parkinson's, Huntington's diseases, Autism spectrum disorder and Angelman syndrome, all characterized by synaptic alterations, are collectively considered as synaptopathies (Baudry et al., 2012; Fogarty, 2019; Taoufik et al., 2018). Therefore, it is particularly interesting to investigate the molecular mechanisms underlying altered synaptic plasticity in these neuropathologies in order to unveil possible common therapeutic targets. In this context, we analyzed 5-HT7R as a potential target. Indeed, its deregulation has been associated with several neurodevelopmental and neurodegenerative diseases (L. Costa et al., 2018; Crispino et al., 2020; De Filippis et al., 2014, 2015; Hashemi-Firouzi et al., 2017; Kelleher & Bear, 2008; Quintero-Villegas & Valdés-Ferrer, 2022). 5-HT7R involvement has been widely demonstrated in synaptic plasticity, since its activation has a positive impact on spinogenesis, synaptogenesis and neurite outgrowth (Canese et al., 2015; Rojas et al., 2014; Speranza et al., 2013, 2017). All these mechanisms depend on local protein synthesis, thus it is possible to hypothesize that 5-HT7R activation at the synapse may be linked to synaptic protein synthesis (Crispino et al., 2020), which is a crucial mechanism underlying synaptic plasticity.

As a first step, we verified the involvement of 5-HT7R in synaptic protein synthesis using synaptosomes isolated from rat cerebral cortex. Synaptosomes, as a model of synapse separated from soma, are a well characterized tool for studying synaptic protein synthesis (Cefaliello et al., 2020; Hafner et al., 2019; Ibrahim et al., 2023; Perrone-Capano et al., 2021).

Stimulation of synaptosomes with LP-211, a selective 5-HT7R agonist, resulted in activation of the ERK pathway, in agreement with previous data showing LP-211-dependent ERK activation in neuronal primary cultures (Speranza et al., 2013). This result confirms the proper activation of the receptor in synaptosomes. To investigate possible effects of 5-HT7R stimulation on synaptic protein synthesis, we studied the level of expression of the eukaryotic initiation factor 2B2 (eIF2B2). This factor was previously demonstrated to be locally synthetized in axons, modulating axonal protein synthesis and axonal growth (Kar et al., 2013). Therefore, we used it as a marker of local activity of protein synthesis. Surprisingly, its synaptosomal level decreased after stimulation of 5-HT7R with the selective agonist, LP-211. To investigate more directly the synaptosomal system of protein synthesis, we used a non-radioactive, puromycin-based, technique named SUnSET (Goodman et al., 2011; Schmidt et al., 2009). When synaptosomal fractions were incubated in the presence of LP-211, we observed a decrease in the total activity of synaptic protein synthesis, confirming our previous results with eIF2B2. These data were quite surprising since stimulation of 5-HT7R is known to enhance overall protein synthesis in neuronal cells (Speranza et al., 2013). This selective and specific inhibition of protein synthesis in synaptic terminals highlights once more the striking association between synaptic plasticity and 5-HT7R activation.

The second step aimed to investigate alterations of synaptic plasticity and the involvement of 5-HT7R, in two different mouse strains of NDDs, such as the BTBR, an ASD model, and the Ube3A$^{tm1Alb/J}$, an Angelman syndrome model.

ASD is linked with several alterations of synaptic plasticity (Hansel, 2019). Indeed, both ASD patients and mice models display abnormal dendritic spine morphologies (Hutsler & Zhang, 2010; Lo & Lai, 2020), and several genetic variants associated with ASD are known to affect dendritic protein synthesis (Lo & Lai, 2020). BTBR mice are a well-known animal model for ASD, exhibiting various autism-like behaviours, such as impaired reciprocal social interactions in both juvenile and adult stages, diminished social approach in adulthood (Bolivar et al., 2007; McFarlane et al., 2008; Moy et al., 2007; Yang, 2007; Yang et al., 2007, 2009), and elevated levels of repetitive self-grooming (McFarlane et al., 2008; Yang, 2007; Yang et al., 2007, 2009). Nowadays, only few studies have focused on the synaptic area of BTBR mice (Wei et al., 2015, 2016). To this aim, we assessed the enrichment of synaptic hallmarks in the synaptosomal fraction from BTBR mouse brain cortex, as compared to WT. The higher enrichment of synaptophysin, a synaptic vesicle marker, in BTBR synaptosomes, as compared to WT, supports the hypothesis of synaptic alterations in BTBR mice.

Several reports correlated ASD defects with alterations of serotonergic signaling (Garbarino et al., 2019; Muller et al., 2016; Rodnyy et al., 2023), with 5-HT7R playing a crucial role (Lee et al., 2021). We focused our attention to the synaptic areas, and identified a reduction in the expression levels of 5-HT7R and diminished ERK phosphorylation levels in BTBR synaptosomes, as compared to WT.

Interestingly, it was suggested that altered mechanisms of synaptic protein synthesis may contribute to ASD (Kelleher & Bear, 2008). To deepen the investigation on this aspect, we first evaluated, in BTBR synaptosomes

compared to WT, the phosphorylation levels of eIF4E, which has been previously shown to be implicated in local translation (Pasciuto & Bagni, 2014). The phosphorylation of eIF4E has been used as index of activation of the synaptic system of protein synthesis (Bramham et al., 2016; Pyronnet, 1999). Therefore, our results showing a decreased phosphorylation of eIF4E, indicated a diminished protein synthesis in BTBR, suggesting that the latter process is impaired in the disease. These results were confirmed using the SUnSET technique with BTBR synaptosomes. Interestingly, stimulation of the 5-HT7R with the agonist LP-211 increased protein synthesis levels in BTBR synaptosomes. This effect was specifically attributed to 5-HT7R, since it was reversed when incubation was performed in presence of LP-211 together with SB- 269970, the antagonist of 5-HT7R.

Altogether, these data indicate that the machinery of protein synthesis located in synaptic territories is selectively impaired in BTBR mice, and that activation of 5-HT7R is able to reverse this effect.

To generalize our results to other NDDs, we used a mouse model for Angelman syndrome, which displays several comorbidities with Autism, including synaptic deficits (Baudry et al., 2012; Hutsler & Zhang, 2010). First, we compared the expression levels of 5-HT7R in AS and WT hippocampal neuronal cultures, and we did not observe any difference. On the other hand, we detected increased 5-HT7R levels in AS mice brain homogenates, as compared to WT, and decreased levels in AS synaptosomes. This altered expression pattern suggests an important role of 5-HT7R in AS disease, and its selective regulation at the synaptic level. As a second step, we evaluated the synaptic activity of protein synthesis in AS synaptosomes, as compared to WT,

and we observed a significant decrease of this activity. On the other hand, no difference in protein synthesis was detected in AS hippocampal neurons compared to WT. These results highlight the notion that alterations selectively affect the system of protein synthesis located in synaptic terminals. More importantly, incubation of AS synaptosomes with LP-211 led to a significant increase in local protein synthesis, which was blocked by coincubation with SB-269970, confirming the rescuing effect of 5-HT7R stimulation. Interestingly, incubation of WT synaptosomes with LP-211 induced a significant reduction in local protein synthesis, which was also observed in rats synaptosomes. Conversely, incubation of WT hippocampal neurons with LP-211 induced an increased protein synthesis, in agreement with previous data (Speranza et al., 2013). Altogether, our findings highlighted for the first time the dual role of 5-HT7R on protein synthesis in neurons, with a positive effect in the overall neuronal system of protein synthesis, and an inhibitory effect at the synaptic level. This apparent discrepancy emphasizes the selective action of 5-HT7R stimulation in synapses, and requires further study to unveil the underlying molecular mechanisms.

It has previously been reported that 5-HT7R activation leads to an increase in the number of dendritic spines in hippocampal neuronal cultures (Speranza et al., 2017), while AS neurons display a deficit in dendritic spine density in various brain areas (Dindot et al., 2007; Sun, Zhu, et al., 2015; Yashiro et al., 2009). Therefore, we evaluated whether 5-HT7R activation in AS neurons could reverse the dendritic spine impairment. Our results confirmed this hypothesis, by showing that prolonged stimulation of 5-HT7R leads to an increase in dendritic spine density in AS neurons, which was partially reversed

by co-incubation with SB-269970. In WT neurons LP-211 also induced an increase in dendritic spine density, while incubation with SB-269970 alone or in combination with LP-211, decreased the spine density, as compared to control, in line with previous evidence (Speranza et al., 2017). These results support the idea that constitutive activity of 5-HT7R is crucial for the establishment of dendritic spines and consequently for synaptic plasticity. Interestingly, the response of AS neurons to SB-269970 is different from that in WT neurons, in that the incubation with the 5-HT7R antagonist led to a significant increase in AS dendritic spine density, although it was lower than the one caused by LP-211. The reason for this different 5-HT7R effect in AS mice compared to WT is not clear yet, but it is in line with previous reports suggesting that, in some cases, both 5-HT7R agonists and antagonists have positive effects on ASD (Lee et al., 2021).

Altogether, these data support our hypothesis that altered synaptic plasticity, and in particular alteration of synaptic protein synthesis, is a common mechanism in different NDDs, and can be reversed by stimulation of 5-HT7R.

Our next goal was to extend these results to neurodegenerative diseases, since emerging evidence suggest the key role played by impaired synaptic plasticity in these neuropathologies (Cefaliello et al., 2020; Fallini et al., 2016; Nijssen et al., 2018). To this aim, we focused our study on EPM1, a neurodegenerative disease characterized by mutation in the CSTB gene. Interestingly, alterations during neuronal development have recently been reported in human and mouse model of EPM1 (Di Matteo et al., 2020). Therefore, the neurodegenerative disease EPM1 displays also some features

of neurodevelopmental diseases, which makes it a very appropriate model in these studies.

Taking advantage of the availability of two different human cell lines for the pathology derived from 2 different EPM1 patients, we demonstrated that EPM1 neurons displayed an altered morphology, with cell bodies similar to controls, but with longer, thinner and more branched neurites. Moreover, we generated hCOs from iPSCs of the two EPM1 patients and controls (Di Matteo et al., 2020), and we were able to characterize alterations in synaptic plasticity in this pathological context, resulting from low CSTB level in this 3D human model. As expected, low levels of CSTB had a strong impact on synaptic physiology. Indeed, in synaptosomes from EPM1 hCOs, the abundance of synaptic proteins was significantly decreased, with protein-specific profiles. In particular, patient synaptophysin levels remained lower than that of the control at all maturational stages, indicating a general impairment at synapses, while syntaxin and synaptotagmin 1/2 returned to control levels at 11 months. Since syntaxin, a t-SNARE protein, and synaptotagmin 1/2, a calcium sensor involved in the exocytosis of synaptic vesicles, are key elements of synaptic activity, our results suggested that the activity of EPM1 synapses is delayed compared to controls. These data are in line with the previous results indicating lower levels of synapsin 1 puncta in developing cerebellum from CSTB knockout mice which were rescued at later stages (Joensuu et al., 2014). To investigate the mechanism underlying alterations of synaptic composition in EPM1 hCOs, we focused our attention to synaptic protein synthesis (Perrone-Capano et al., 2021). In particular, we analyzed the synaptosomal expression levels of eIF4G2, an initiation factor that, as eIF2B2

is axonally synthesized (Kar et al., 2013). We observed a relevant decrease of eIF4G2 in EPM1 synaptosomes, suggesting the impairment of synaptic protein synthesis in the pathology. On the other hand, eIF4G2 protein was more abundant in EPM1 homogenates in comparison with the control at early maturation stages, while it returned to control levels at later maturational stages. These data suggest that one of the pathological features of the disease during neuronal development is the alteration of transport mechanisms of different cargos, including translation-related proteins, from the soma to the synaptic areas of the neurons. This hypothesis is supported also by the altered intracellular transport and protein synthesis machinery described in cerebellar synaptosomes from CSTB-KO mice (Gorski et al., 2020). It is noteworthy that CSTB is one of the proteins synaptically synthesized (Penna et al., 2019). Therefore, the impairment of the synaptic protein synthesis in EPM1 may aggravate the CSTB deficit specifically in the synaptic area. In synaptosomes from EPM1 hCOs we also observed a decreased expression level of the EVs marker CD81. The results from Mass spectrometry analyses corroborated these findings, showing depletion of proteins involved in secretion and vesicles transport in synaptosomes from EPM1 hCOs. These data suggest two possible scenarios related to the pathological low levels of CSTB in EPM1: i) lack of CSTB alters the secretion mechanism and trafficking of EVs in synaptic areas and ii) lack of secreted CSTB affects extracellular neurogenic environment leading to the pathology. In conclusion, our results suggest that EPM1 is characterized by early neuronal differentiation, but delayed synaptic functions, which leads to synaptopathy and altered neuronal

morphology and circuitry, which could be responsible for the epileptic phenotype.

CSTB is a particularly interesting protein since it is locally synthesized in rat brain synaptosomes and is secreted into the extracellular medium by a depolarizing-dependent mechanism (Di Matteo et al., 2020; Penna et al., 2019). Thus, it could participate in the molecular mechanisms underlying synaptic plasticity. Since we demonstrated that 5-HT7R stimulation regulates synaptic protein synthesis, we evaluated CSTB expression levels in synaptosomes from rat brain incubated with LP-211. We found that stimulation of 5-HT7R resulted in a decrease in CSTB expression, indirectly suggesting a possible inhibitory role of 5-HT7R on local protein synthesis. At the same time, 5-HT7R stimulation increased CSTB release from synaptosomes both in free form and associated to EVs fraction.

To further elucidate CSTB release mechanisms, we incubated rat cerebral cortex synaptosomes in different experimental conditions by modifying extracellular and intracellular calcium ions concentrations. We first investigated synaptosomal CSTB level and demonstrated that its decrease, in depolarizing conditions, was calcium-dependent because it was prevented by the absence of calcium in the extracellular medium. Under depolarizing conditions, calcium channels in presynaptic terminals open, leading to a physiological increase in intracellular calcium levels. Thus, the CSTB decrease that we observed in this condition, depends on the transient increase in intracellular calcium concentration. It is noteworthy that the depolarization-dependent CSTB release mediated by this transient increase of calcium concentration only affects the soluble form, while the EVs-associated form

release is calcium-independent. Altogether, these results indicate that CSTB release occurs with two different modalities, which are differently regulated.

The influence of calcium ions in regulating CSTB secretion was demonstrated in different experiments where we modified the intracellular calcium concentrations, outside the physiological range, increasing them with ionomycin or decreasing them with BAPTA. With this experimental paradigm, CSTB release, both as soluble protein and as EVs-associated form, significantly decreased, demonstrating that intracellular calcium concentration has to remain within a physiological range to ensure proper CSTB release.

The dual release mechanism of CSTB as a soluble protein *or as* EVs-associated was also demonstrated in synaptosomes from embryonic mouse brain and hCOs, confirming the relevance of CSTB release from synapse during neuronal development.

In general, the demonstration of CSTB release from synapses in two different forms, with different regulation mechanism opens new perspectives on the biological role of CSTB as a long- and short-distance signaling cue. Indeed, it is well known that EVs secreted by neurons modify the neurogenic niche to make the microenvironment more appropriate for neurogenesis and circuit assembly. Accordingly, altered EVs secretion has a functional relevance to neurodevelopmental diseases (Ma et al., 2019; Sharma et al., 2019). In this context, data indicating that secreted CSTB is able to remodel the extracellular space for neurons by promoting tangential migration and recruitment of interneurons are particularly relevant (Di Matteo et al., 2020).

7. CONCLUSIONS

The main aim of this work was to investigate the possibility that synaptic plasticity alterations represent common mechanisms in both neurodevelopmental and neurodegenerative diseases. We demonstrated that synaptic protein synthesis is altered in two model of neurodevelopmental disorders (ASD and AS), and in one model of neurodegenerative disease (EPM1). IN EPM1 we also showed alterations of EVs secretion from synapses. Interestingly, 5-HT7R emerged as a new therapeutic target, and its agonists may represent a general therapeutical approach for synaptopathies, pathologies of the nervous system characterized by altered synaptic plasticity.

8. GRAPHICAL SUMMARY

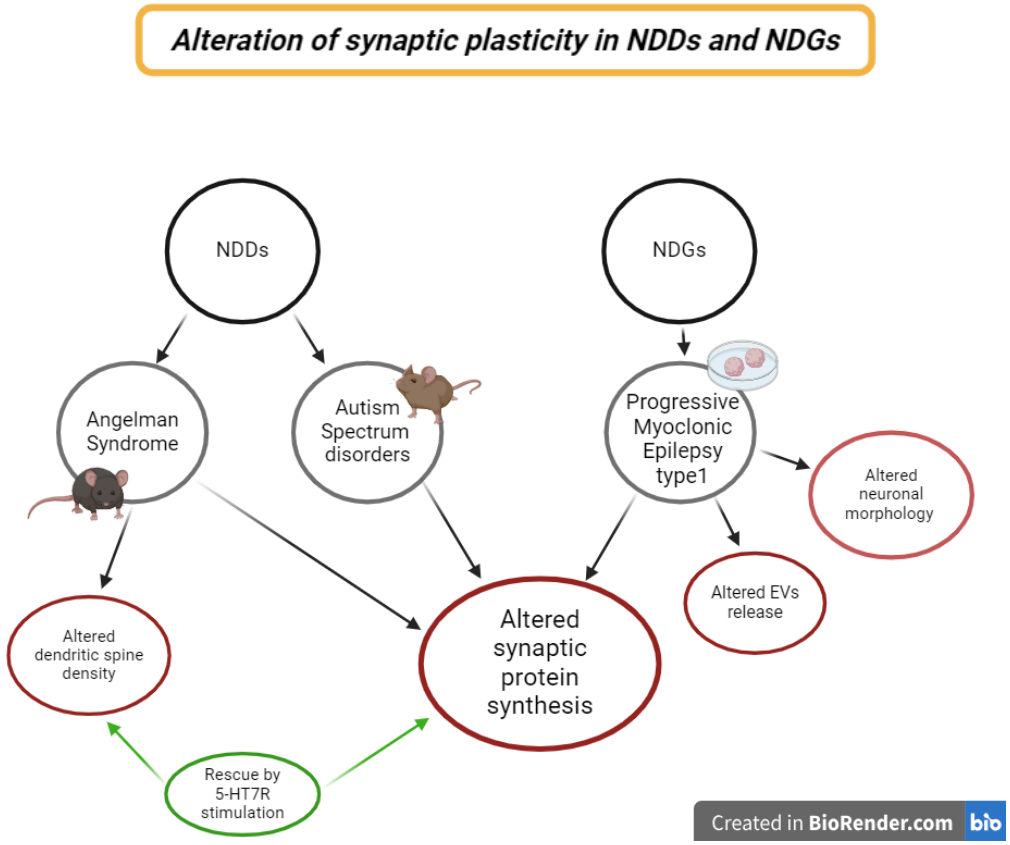

Figure 24: Alteration of synaptic plasticity in neurodevelopmental (NDDs) and neurodegenerative (NDGs) diseases. *Concept map showing impaired synaptic plasticity mechanisms in models of NDDs and NDGs. Interestingly, synaptic protein synthesis is a commonly altered mechanism in these models. Stimulation of 5-HT7R is able to rescue the deficit of dendritic spine density and synaptic protein synthesis in NDDs.*

9. ACRONYMS

5-HT *serotonin*
5-HT7R *serotonin receptor 7*

AD *Alzheimer's disease*
Arc *activity-regulated cytoskeletal protein*
AS *Angelman syndrome*
ASD *Autism spectrum disorder*

BDNF *brain-derived neurotrophic factor*

CNS *central nervous system*
CSTB *cystatin B*

eIF2B2 *eukaryotic initiation factor 2B2*
eIF4E *eukaryotic initiation factor 4E*
eIF4G2 *eukaryotic initiation factor 4G2*
EPM1 *Myoclonic epilepsy type 1*
EVs *extracellular vesicles*

FXS *fragile X syndrome*

KO *knock-out*

hCOs *human cerebral organoids*
HD *Huntington's disease*

ILVs *intraluminal vesicles*
iPSCs *induced pluripotent stem cells*

LTD *long term depression*

LTP *long term potentiation*

MVBs *multivesicular bodies*

NDDs *neurodevelopmental diseases*
NDGs *neurodegenerative diseases*

NPCs *neuronal progenitor cells*

PD *Parkinson's disease*

SERT *5-HT transporter*
SUnSET *surface and sensing of translation*

TrkB *Tropomyosin receptor kinase B*

WT *wild-type*

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