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Title:

Removal of the calcium-dependent regulation of ATP binding in Synapsin I has distinct effects at excitatory and inhibitory synapses

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CONTENTS

1. SUMMARY

Synapsins are the most abundant family of neuro-specific phosphoproteins associated with the cytoplasmic surface of the synaptic vesicle membrane. These proteins actively regulate synaptic transmission at the level of the presynaptic terminal by controlling the storage and mobilization of synaptic vesicles within a reserve pool. However, it is hypothesized that synapsins could be involved in other stages of synaptic vesicle dynamics such as trafficking, docking, fusion with the plasma membrane and consequent recycling. Synapsin I (SynI) in particular is expressed two isoforms (Ia and Ib) at the presynaptic compartment of all neurons in the adult brain. Several studies suggest that SynI is also involved in axon elongation and synaptic vesicle fusion kinetics. In human, nonsense and missense mutations of SYN1 gene are related to several diseases such as epilepsy and autism spectrum disorder; in fact, SynI knockout (KO) mice show an epileptic and autism-like phenotype. To carry out its functions, SynI requires to bind ATP in a Ca^{2+}-dependent manner thanks to the coordination of a glutamate residue (E373). As ATP binding regulates SynI oligomerization and SV clustering, we analyzed the effect of E373K mutation on neurotransmitter release and short-term plasticity in excitatory and inhibitory synapses. We coupled electrophysiology (patch-clamp recordings) with electron microscopy in primary SynI KO hippocampal neurons in which either the human wild type or the E373K mutant SynI were re-introduced by infection with lentiviral vectors. Our data indicate that E373K mutation affects predominantly excitatory synapses. The frequency of miniature excitatory postsynaptic currents (mEPSCs) was enhanced, without changes in the amplitude and in the number of excitatory synapses. The increment of mEPSCs frequency was totally abolished after acute injection of BAPTA-AM (a specific Ca^{2+} chelator), suggesting a possible

alteration of Ca^{2+} homeostasis at the presynaptic terminal. Excitatory E373K-Syn I neurons showed reduced evoked EPSC amplitude attributable to a reduction of the readily releasable pool (RRP), while, on the contrary, inhibitory E373K-Syn I neurons did not show any difference both in miniature, evoked IPSC amplitude and RRP size. While no effects in the dynamics and steady state of depression were detected, both excitatory and inhibitory E373K-Syn I neurons failed to recover after stimulation with long high-frequency trains. No mutation-induced changes were observed in network firing/bursting activity as determined with multi-electrode extracellular recordings. Our data suggest that the Ca^{2+}-dependent regulation of ATP-binding to SynI plays important roles in spontaneous and evoked neurotransmitter release that differentially affect the strength of excitatory and inhibitory transmission.

2. INTRODUCTION

2.1. SYNAPSE: STRUCTURE AND FUNCTION

Synapses are very specialized subcellular structures deputed to transfer and storage of information between neurons in the nervous system. "*Synapse*" is the Anglicization of the Greek word "*Synapsis*", coined by Sir Charles Sherrington in 1897 to describe the anatomical structure that functionally links two communicating neurons. Although at that time the Neuron Doctrine was still a contentious issue, Sherrington clearly described synapses as a discontinuous intercellular junction, laying down the bases for understanding of nervous system functioning (Levine (2007). Both in vertebrates and in invertebrates, synapses can be divided in two types: electrical and chemical synapses (Fig. 1). Electrical synapses are essentially formed by so called "gap junctions", clusters of transmembrane proteins (connexins) that enable direct communication between two adjacent cells. This type of synapse can mediate signal transmission between cells via ion flux (Bennett and Zukin 2004, Connors and Long 2004). However, in vertebrates, the most common mechanism for signaling is through chemical synapses. Neurotransmitter-operated chemical synapses present a typical asymmetrical organization, characterized by the presence of two different compartments: a presynaptic and a postsynaptic terminal specialized for a distinct signaling role. The main function of the presynaptic terminal is to receive electrical stimuli (action potentials) and convert them into a chemical signal (neurotransmitter release); conversely, the main function of the postsynaptic terminal is to receive this signal and convert it into an electrical and/or metabolic change of the postsynaptic neuron. This phenomenon is known as synaptic transmission, a complex and modular process that is tightly regulated at the level of the synapse itself (Bear, Connors et al. 2016).

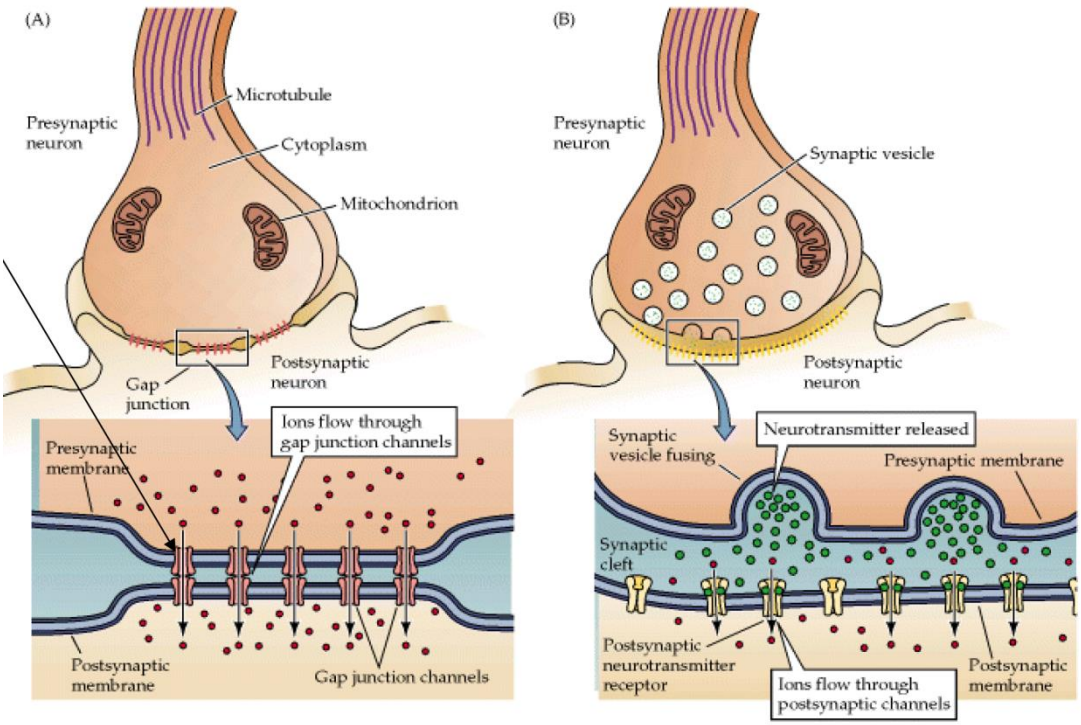


Figure 1. Two modalities of synaptic transmission: electrical (left) and chemical (right) synapses. (A) Schematic representation of an electrical synapse: transmission is mediated by clusters of intercellular proteins called gap junctions that connect two adjacent cells, permitting the bidirectional passage of ions and small metabolites. **(B)** schematic representation of a chemical synapse: transmission requires a complex of presynaptic and postsynaptic machineries. At the presynaptic compartment, the arrival of action potential permits the activation of voltage-gated calcium channels (VGCCs) with a consequent Ca^{2+} influx that regulates neurotransmitter release. At the postsynaptic compartment, specific receptors are capable of detecting and translating the presynaptic message into various postsynaptic events (Bear et al. 2016).

2.2. CHEMICAL SYNAPSES

Chemical synapses can generally be divided into two groups: excitatory and inhibitory.

Excitatory synapses promote membrane depolarization and action potential generation. Their presynaptic compartment is characterized by the presence of synaptic vesicles (SVs) containing excitatory neurotransmitters (mainly glutamate). The postsynaptic compartment, located in the dendrites (dendritic spines), presents neurotransmitter receptors; in the case of glutamate, the two most abundant receptors in the central nervous system are α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA-R) and *N*-methyl-D-aspartate receptors (NMDA-R). When glutamate is released into the synaptic cleft, it binds receptors that mediate the influx of Na^+ and favor the generation of action potentials (Sudhof 2004, Schoch and Gundelfinger 2006, Jin and Garner 2008). Inhibitory synapses, on the contrary, reduce the probability of generating of an action potential by hyperpolarizing the postsynaptic membrane. µ-aminobutyric acid (GABA), and secondly glycine, are the two most abundant inhibitory neurotransmitters in the adult brain. Both of these transmitters directly activate distinct, but homologous, classes of receptors: the GABA A receptor and the Gly receptor. Both of them are chloride-permeable ion channels that mediate the influx of Cl^- shifting the membrane potential to more negative values (Rivera, Voipio et al. 1999).

2.3. SYNAPTIC TRANSMISSION

Synaptic transmission is a complex process by which two neurons communicate. It starts at the presynaptic terminal when one or more action potentials, initiated in the neuronal cell body, trigger neurotransmitter release. The change in membrane voltage, due to the action potential, induces the opening of voltage-gated calcium channels (VGCCs), mediating the transient influx of Ca^{2+} in the presynaptic terminal. Ca^{2+}, acting as a second messenger, binds SNARE complex proteins, triggering SV docking, fusion with the plasma membrane, SV exocytosis and, as a direct consequence, neurotransmitter release in the synaptic cleft. After exocytosis, SVs undergo endocytosis, recycle and refill with neurotransmitters, ready for a new cycle (Goda and Sudhof 1997). In the presynaptic compartment, the relationship between action potentials and the neurotransmitters release is strictly mediated by intracellular second messengers and extracellular modulators.

A fundamental role is played by SVs that are involved in aspect of synaptic transmission. The SV trafficking cycle that occurs at the presynaptic terminal can be subdivided into 5 sequential steps (Fig.2). First of all, SVs have to be filled with the neurotransmitters (step 1) and then clustered in the proximity of the active zone (step 2). They have to dock at the active zone (step 3) and primed (step 4) to permit the binding of Ca^{2+} to SNARE complex proteins and the fusion-pore opening (step 5). After fusion, SVs undergo endocytosis and recycling (step 6). These important steps occur in at least three alternative pathways. The first one is called “kiss-and-stay”, in which vesicles are acidified and refilled with neurotransmitters without undocking, permitting the formation of the readily releasable pool (RRP). The second one is called “kiss-and-run”, in which vesicles, after reacidification and refilling, are undocked and can start the cycle again. The last possible pathway involves

endocytosis via clathrin-coated pits, passage through an endosomal intermediate, reacidification and refill (Waites and Garner 2011).

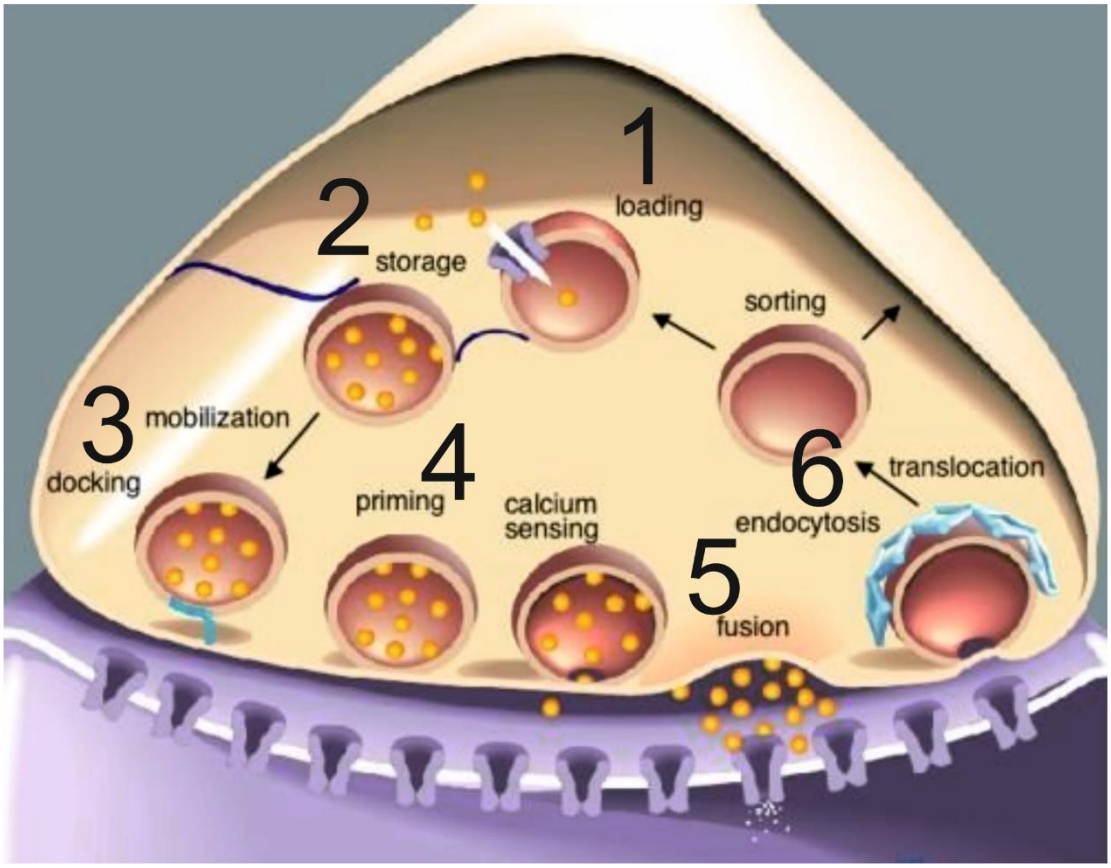


Figure 2. Synaptic vesicle cycle. Representative scheme of SV dynamics. SVs are loaded with neurotransmitter through specific transporters (step1). They translocate to the active zone (step2) where they selectively dock (step3) and prime, becoming fusion competent (step4). Calcium influx triggers SV fusion and release of neurotransmitter into the synaptic cleft (step5). After fusion, vesicle proteins and membrane are retrieved by clathrin-mediated endocytosis (step6) (Richmond 2005).

2.3.1. Synaptic vesicle pools

SV pools are an important determinant of synapse physiology and play a fundamental role in determining the efficacy of short-term plasticity. Several authors considered it necessary to divide SVs into different physical/functional pools, even if there is no universally accepted categorization (Rizzoli and Betz 2005, Schweizer and Ryan 2006). Betz and collaborators conventionally distinguish three different pools: the readily releasable pool (RRP), the recycling pool and the reserve or resting pool (RP) (Stevens and Williams 2007). RRP is represented by a class of SVs that are immediately available and readily discharged after 1-2 seconds at 40Hz stimulation. The size of the RRP can be determined in two ways, applying an extracellular solution of hypertonic sucrose (Rosenmund and Stevens 1996) or evaluating the cumulative curve of evoked postsynaptic current amplitude after a 40Hz tetanic stimulation for 1 second (Fioravante and Regehr 2011). After the stimulation, RRP is completely depleted and, if the stimulus continues, new SVs are recruited from the recycling pool in physiological conditions. Rizzoli and Betz proposed the existence of another pool called RP, which includes vesicles that can be released only during bouts of intense, often "non-physiological" stimulus (Rizzoli and Betz 2005).

2.3.2. Role of calcium ions

Neurotransmitter release is a stochastic event; electrical presynaptic stimuli generated by action potentials do not always result in an actual release. Furthermore, rare spontaneous release of neurotransmitters could happen without nervous stimulation. For this reason, it is necessary to introduce the concept of "release probability" (Pr), defined as the probability that a quantum of neurotransmitter is released in response to an electrical stimulus. Ca^{2+} influx considerably enhances the Pr by activating Ca^{2+} sensors. As a consequence, VGCCs play an important role in

the regulation of the Pr, allowing Ca^{2+} to interact with the fusion machinery (Augustine 2001). Ca^{2+} influx at the presynaptic terminal could generate three different types of release. The first one is commonly defined "evoked or synchronous release" and is characterized by very rapid exocytosis of SVs already docked or located next to the active zone. The quick release is due to the large amount of Ca^{2+} that enters the presynaptic compartment following the action potential. However, the persistence of Ca^{2+} at very low concentration could induce a very slow SVs release, conventionally called "asynchronous release" (Hagler and Goda 2001, Yoshihara, Adolfsen et al. 2003). Ca^{2+} influx, mediated by VGCCs, represents an essential step for neurotransmitter release. As a direct consequence, biochemical modification of VGCC activity can, in principle, regulate synaptic transmission. In vertebrates, synaptic transmission is mediated by a group of VGCCs titled CaV2, in particular P/Q- and N-type channels (Eggerman et al. 2012). Each of them can directly bind several members of the SNARE protein complex such as syntaxin 1, SNAP-25 and synaptotagmin 1 (Wheeler, Sather et al. 1994, Wu, Westenbroek et al. 1999). The third type of release is the so called "spontaneous release". It is not associated with APs and, in fact, it is recorded in presence of tetrodotoxin (TTX), a voltage-gated Na^{+} channels blocker. However, it has been demonstrated that a fraction of spontaneous release is Ca^{2+}-dependent (Neher and Sakaba 2008).

2.4. SYNAPTIC PLASTICITY

Synaptic plasticity could generally be defined as the intrinsic capability of the brain to change and adapt in response to new information. More specifically, plasticity refers to the ability, at preexisting synapses, to modify synaptic transmission in terms of strength or efficacy. In the last decades, it has been supposed that synaptic plasticity could be the physiological mechanism at the base of learning and memory and could play a key role in the development of neuronal circuits. In fact, alterations in synaptic plasticity during development are associated with several neuropsychiatric disorders. We can traditionally distinguish two sub-types of synaptic plasticity: short-term (STP) and long-term (LTP) plasticity. In general, STP occurs in a timescale of tens of milliseconds to a few minutes and is restricted to the synapse, without inducing any changes at the level of the soma. LTP, on the contrary, could last from minutes to hours and involves not just the synapses per se, but also the soma, inducing modifications in gene expression (Citri and Malenka 2008).

2.4.1. Short-term synaptic plasticity

Previous studies identified several types of STP, each characterized by a timescale from tens of milliseconds to a few minutes, in every synapse in every animal, from simpler invertebrates to mammals. These types of STP play an important role in short-term adaptation and in several forms of short-term memory (Zucker and Regehr 2002). A common characteristic of all types of STP is the large transitory accumulation of Ca^{2+} at the presynaptic compartment, triggered by high frequency neuronal network activity. This presynaptic accumulation directly induces changes in Pr and more generally in the biochemical processes at the base of SV exocytosis. The three most common forms of STP are depression, facilitation and augmentation/post-

tetanic potentiation (PTP) (Fig.3). Katz and Miledi (1968) demonstrated that if a presynaptic neuron was stimulated by two pulses given in a short time interval (from tens to hundreds of milliseconds), the second response was different from the first one, depending on the Pr. When the second evoked response is smaller than the first one, we are in the presence of depression; conversely, when the second evoked response is larger than the first one, we are in the presence of facilitation (Katz and Miledi 1968). A sustained high frequency presynaptic stimulation normally generates an enhanced response that can last from tens of seconds to several minutes; this phenomenon is called augmentation or PTP (Fisher, Fischer et al. 1997).

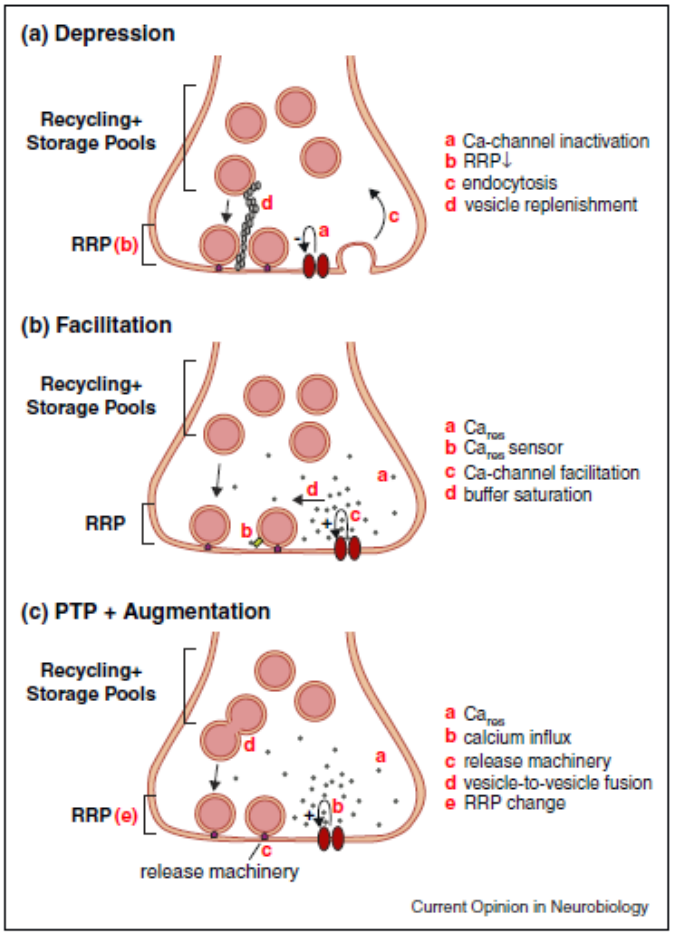


Figure 3. Presynaptic forms of short-term plasticity. Schematic image illustrating the proposed mechanisms for (A) depression, (B) facilitation, and (C) post-tetanic potentiation (PTP) augmentation (Fioravante and Regehr 2011).

2.4.2. Presynaptic role of calcium in STP

The calcium ion is one of the most important second messengers and plays a key role in several forms of synaptic plasticity. Its best known function is to bind multiple affinity sites on synaptotagmin 1, part of the SNARE complex, allowing the fusion of SVs with the plasma membrane. SV fusion and release is mediated by high Ca^{2+} concentration (about 10-100 nM), in the proximity of VGCC localization in the plasma membrane of the synaptic bouton (Schneggenburger and Neher 2005). The strength and magnitude of SV release is due to the distance between open channels and the active zone. High affinity calcium-binding proteins can rapidly reduce free Ca^{2+} concentration, preventing calcium binding with SNARE complex proteins and affecting the release probability (Roberts 1993). After SV release and calcium dispersion in the presynaptic compartment, the residual Ca^{2+} is gradually extruded.

2.5. SYNAPSINS:

2.5.1. Genes and proteins

Synapsins are the most abundant family of neuro-specific phosphoproteins associated with the cytoplasmic surface of the SV membrane (Fig. 4) (Goelz et al. 1981). These proteins actively regulate synaptic transmission at the presynaptic terminal by controlling the storage and mobilization of SVs. However, it has been shown that synapsins could be involved in other stages of synaptic vesicle dynamics such as trafficking, docking, fusion with the plasma membrane and relative recycling (Cesca et al. 2010). This family is comprised of ten isoforms termed SynI (a-b), SynII (a-b) and SynIII (a-f) (Sudhof et al. 1989, Porton et al. 1999). These isoforms are generated in mammals by alternative splicing of three distinct genes: SYN1, SYN2 and SYN3, mapped respectively on human and mouse chromosome X (Xp11), chromosome 3 (3p25) and chromosome 22 (22p12.3) (Yang-Feng et al. 1986, Li et al. 1995, Hosaka and Sudhof 1998, Kao et al. 1998). All isoforms are characterized by the combination of different domains: at the N-terminal region are present three domains (A-C) that are highly conserved and at the C-terminal region the remaining domains (D-J) that are differently distributed between isoforms (Song and Augustine 2015). SynI is expressed in all its isoforms at the presynaptic terminal of all neuronal cells in the adult brain (Ferreira et al. 1998). Several studies demonstrate that SynI is involved in axon elongation and SV fusion kinetics (Coleman and Bykhovskaia 2009). Both isoforms are associated with synapse formation and neurotransmitter release at inhibitory synapses (Ferreira et al. 1998). Recent studies suggest that SynI can phase-separate out of solution in physiological conditions. It has been already described that lipid vesicles and other SV cluster components specifically partition into these condensates, suggesting that SynI phase separation guides SV cluster

formation, generating a reservoir for various synaptic proteins (Milovanovic et al. 2017). Calcium/calmodulin-dependent protein kinase II (CaMKII) phosphorylates SynI, permitting SV cluster release (Benfenati et al. 1992); in fact, the SynI condensates dissolve when CaMKII and ATP are added (Milovanovic et al. 2018). Although SynII shows similar functions to SynI, such as axon elongation and synapse formation (Ferreira and Rapoport 2002), many studies suggest that this protein could have a crucial role in SV docking, regulating synaptogenesis and the formation of the vesicular reserve pool at excitatory synapses (Han et al. 1991, Ferreira et al. 1995, Gitler et al. 2008). SynIII is exclusively expressed in the early stages of neuronal development and almost absent in adult brain. Its core function is to actively regulate synaptogenesis and neurogenesis, mediating process elongation and differentiation (Ferreira et al. 2000). Total deletion of SynIII causes an abnormal neurotransmitter release only at inhibitory synapses, leaving apparently unchanged excitatory ones (Feng et al. 2002, Porton et al. 2011).

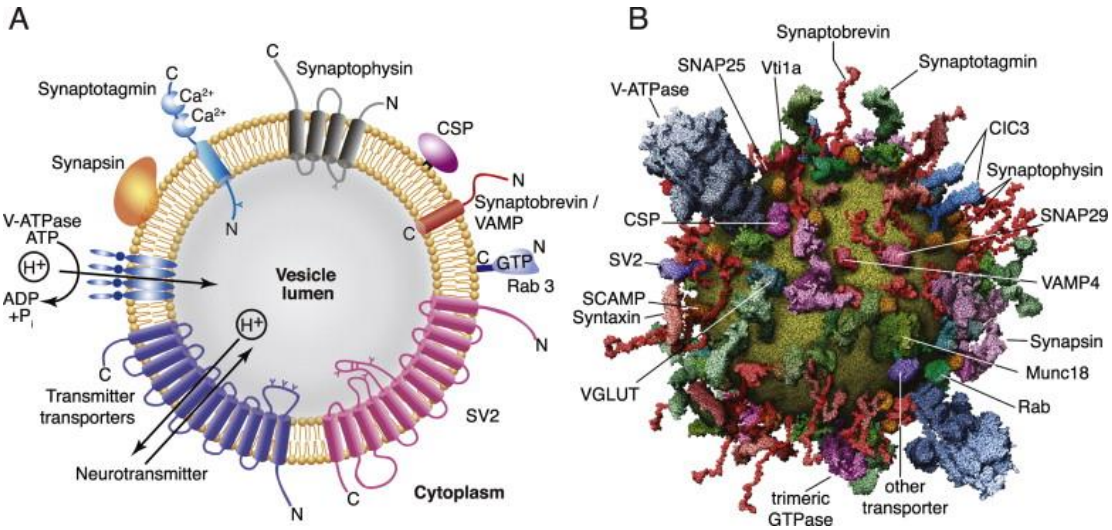


Figure 4. 3D schematic model of an excitatory synaptic vesicle (Zucker et al., 2014)**.**

2.5.2. Synapsin domains

Given the importance that the different domains play in determining the function of synapsins, it is necessary to summarize what is known about all domains (Fig.5).

Domain A is one of the most conserved domains, and is known to interact directly with the phospholipid bilayer of SVs. cAMP-dependent protein kinase (PKA) and Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI) can neutralize this interaction by phosphorylation of a serine residue (Hosaka and Sudhof 1999). From a physiological standpoint, domain A is involved in the regulation of glutamate release in excitatory synapses (Hilfiker et al. 2005). Domain B is currently considered a linker between domains A and C. Its real function is still unclear but it is known to contain a phosphorylation site for the mitogen-activated protein kinase (MAPK)/Extracellular signal-regulated kinase (Erk) (Jovanovic et al. 1996). Domain C is the most conserved region across synapsins and is involved in several functions of the protein. It mediates the binding between synapsins and SVs by partially inserting into the phospholipid bilayers of SVs and also mediates homo / hetero dimerization. C-domains of both SynI and SynII present high affinity ATP-binding sites that exhibit similar ATP affinities but ATP-binding to SynI is regulated in a Ca^{2+}-dependent manner. In SynI, the Ca^{2+} binding is mediated by a glutamate residue (Glu373) at a position where SynII contains a lysine residue (Hosaka and Sudhof 1998). Several studies suggest that ATP binding to domain C could play a role in pre/post docking and clustering of synaptic vesicles in inhibitory synapses (Orlando et al. 2014). Domain D, common only to SynI, is a proline and glutamine rich domain. It binds to SH3-domain containing proteins, such as c-Src, Grb2, PI3K, PLC-r and amphiphysin-I and II, as well as CaMKII and Rab3 (Giovedi et al. 2004) and contains two CaMKII phosphorylation sites, involved in glutamate release (Llinas et al. 1991, Chi et al. 2003). Domain D is also known to be implicated in the inhibition

of SynIb targeting to SVs (Gitler et al. 2004). Domain E is typical to synapsin isoforms SynIa, IIa and IIIa. Its best known function is to mediate SynI oligomerization and cross-link synaptic vesicles (Hilfiker et al. 1998, Hilfiker et al. 2005, Monaldi et al. 2010). Domain G is present specifically in SynII; domain H is present only in SynIIb; domains F and I are typical of isoforms "b". They are all proline rich domains and their function is still unknown. Domain J is unique to SynIIIa and could be involved in dopamine and GABA release (Hosaka and Sudhof 1998, Kao et al. 1998, Porton et al. 2004).

Figure 5. Summary of domain structures of mammalian synapsins (Cesca et al. 2010)**.**

2.5.3. ATP-binding to synapsins

All synapsins isoforms display highly conserved ATP-binding modules, localized in the central C-domain. In 1998, Esser and collaborators determined a crystal structure of C-domain (SynC) monomer of bovine SynI, consisting of the 110-420 residues. They suggested that SynC monomers act as an ATP-utilizing enzymes, such as D-ala:D-ala ligase and glutathione synthetase. Despite C-domain is highly conserved, the binding of ATP is differently regulated between the three main Syn isoforms. ATP-binding to SynI is strictly dependent on the coordination with Ca^{2+}. In their work, Esser and collaborators demonstrated, through the crystal structure, that SynC has a specific preference for Ca^{2+} over Mg^{2+} (Esser et al. 1998). In the same year, Hosaka and Sudhof displayed that in SynI the Ca^{2+} recruitment is due to a single and evolutionary conserved glutamate residue (Glu373). On the contrary, SynII does not need the presence of Ca^{2+} for its function and interestingly presents a lysine residue at the same position in which SynI coordinates with Ca^{2+}. The exchange of GluE373 with a lysine converts SynI into a Ca^{2+}-independent ATP-binding protein, exactly as SynII. In recent years, the idea that Ca^{2+} essential for the ATP-binding to SynI has been questioned. Orlando and collaborators in 2014 proposed a computational model of ATP-binding to SynI. Using molecular dynamics (MD) simulation, they demonstrated that the binding is mediated by a conformational transition of a flexible loop (multifunctional loop, MFL) that opens to make the site accessible. This changing in conformation is not affected by the absence of Ca^{2+} and ATP-binding also occurs under free Ca^{2+} conditions. However, they displayed that Ca^{2+} favors SynI tetramer formation at the expence of dimers. In the same work, the authors proposed that ATP-binding to SynI plays an important role in the modulation of SVs clustering and plasticity of inhibitory synapses (Orlando et al. 2014). The role of ATP-binding in SynII function is not completely understood. It is known that

ATP-binding to SynII does not require the presence of Ca^{2+} (Hosaka and Sudhof, 1998). Interestingly, the prevention of the ATP-binding to SynII, induced by a specific mutation (K270Q), compromises the recruitment of SVs after a sustained stimulation (Shulman et al. 2015). ATP role in SynIII function is not known. The only clear aspect is that SynIII shows a dramatically different behavior compared to SynI. While SynI requires Ca^{2+}, SynIII is inhibited by Ca^{2+}. In 1998, Hosaka and Sudhof proposed that in vertebrates the expression of three distinct synapsins regulate synaptic transmission, utilizing ATP but in a specialized way depending on Ca^{2+} (Hosaka and Sudhof, 1998).

2.6. PRESYNAPTIC ROLE OF SYNAPSINS

2.6.1. Synapsin function in invertebrates

Invertebrates present notably only one synapsin gene. In *C. elegans*, the synapsin gene encodes for a single protein, even if two alternatively spliced products have been identified (Kao et al. 1999; Candiani et al. 2010). In *Drosophila*, only one gene has been characterized, encoding six isoforms widely expressed at the level of synaptic terminals (Klagges et al. 1996, Candiani et al. 2010). Studying the neuromuscular junction of synapsin KO Drosophila, Akbergenova and Bykhovskaia suggested that synapsin regulates the maintenance of the RP, the segregation of SVs between the recycling and the RP and the SVs mobilization during sustained high frequency stimulation (Akbergenova and Bykhovskaia 2007). Other invertebrates, with a more complex nervous system, present similarly only one synapsin gene. Squid expresses two distinct synapsin isoforms (Hilfiker et al. 1998) and one synapsin gene has been also found in the snails Aplysia and Helix (Cibelli et al. 1996).

Previous studies in squid giant synapses displayed that the injection of a dephosphorylated form of SynI decreased the amplitude of postsynaptic potentials and inhibited organelle movement along microtubules in extruded axoplasm. In this model, opposite effects have been reported by injection of CaMKII (Llinas et al. 1985, McGuinness et al. 1989, Llinas et al. 1991). Neurotransmitter release resulted inhibited also after presynaptic injection of a peptide corresponding to the highly conserved region of domain E, without affecting the docked SV density (Hilfiker et al. 1998).

In cholinergic synapses of *Aplysia californica*, the injection of an antibody specific to snail synapsins induced an alteration in neurotransmitter release. The evoked postsynaptic currents (ePSCs) rise time resulted significantly slowed, while decay

time and mean amplitude resulted unchanged. Paired-pulse facilitation (PPF) at physiological Ca^{2+} concentrations was decreased, but lowering the Ca^{2+}/Mg^{2+} ratio revealed that PPF was not affected by synapsin neutralization. PTP was strongly reduced and post-tetanic depression (PTD) resulted accelerated (Humeau et al. 2001). Interestingly, PTP inhibition was enhanced upon treatment with lethal toxin (LT, from *Clostridium sordellii*). This effect is possibly due to an activity-dependent increase in the number of active release sites, thus suggesting that synapsins could act at a post-docking level (Humeau et al. 2007).

2.6.2. Synapsin knock out mice model

The models traditionally used for studying the functions of synapsins are knockout mice (Syn KO). Five different mice lines have been generated: SynI KO, SynII KO, SynIII KO, SynI/II double KO and SynI/II/III triple KO. All these mice are perfectly viable and fertile and have a normal life expectancy. They do not present any defects in terms of brain size and gross structure. Every model (except for SynIII KO) is prone to seizures and presents an epileptic phenotype (Li et al. 1995, Etholm and Heggelund 2009). This phenotype is not surprising, since synapsins are involved in correct neuronal maturation, SV trafficking and more generally in synaptic transmission. At the subcellular level, the ablation of synapsins leads to a disruption of the SV pool and distribution, with a consequent alteration in neurotransmitter release dynamics. However, each synapsin has specific and distinct effects on synaptic transmission and seems to differentially affect excitatory and inhibitory synapses (see below).

2.6.3. Glutamatergic transmission

It has been reported that alteration of synapsin function significantly affects glutamatergic transmission. It has been demonstrated that total ablation of the entire SYN1 gene in excitatory neurons enhances evoked excitatory postsynaptic current (eEPSC) amplitude without affecting miniature excitatory postsynaptic current (mEPSC) amplitude in primary cultures of cortical neurons. It seems that this effect is entirely attributable to an increased size of the readily releasable pool (RRP) (Chiappalone et al. 2009). A similar phenomenon has been observed in hippocampal SynI/II/III KO brain slices (Farisello et al. 2013). An identical effect in excitatory transmission could be induced in wild-type (WT), (but not KO) Schaffer collateral-CA1 pyramidal cell synapses by blockade of GABAB receptors, suggesting that the increased strength of excitatory transmission could depend on an impairment of GABAB presynaptic inhibition (Valente et al. 2017). However, the lack of presynaptic GABAB inhibition does not fully explain the potentiation of the excitatory transmission and a further mechanism able to enhance the glutamatergic release could be involved. An interesting recent report suggests that Ca^{2+} channels could play a role in this phenomenon. In fact, analyzing synapsin function in an invertebrate model (*Helix,* land snail), it has been found that the acute silencing of synapsin increases serotoninergic neuron excitability. The authors demonstrated that the hyperexcitability is related to an increase of Ca^{2+} influx through voltage-gated Ca^{2+} channels and an increase of Ca^{2+}-dependent BK currents (Brenes et al. 2015).

2.6.4. GABAergic transmission

In contrast to glutamate release, the effect of synapsins deletion on the GABA release is characterized by a completely different physiological phenotype. Evoked inhibitory postsynaptic current (eIPSC) amplitude is significantly reduced after the

ablation of SynI in primary cultures of cortical neurons and in SynI/II/III KO brain slices (Baldelli et al. 2007, Chiappalone et al. 2009, Farisello et al. 2013). This reduction is associated with a reduction of SVs docked at the active zone (Gitler et al. 2004). Specifically analyzing eIPSCs evoked in SynII KO brain slices, a reduction of the asynchronous GABA release has been observed associated with a loss of tonic inhibition. On the other hand, synchronous GABA release is enhanced (Medrihan et al. 2013). This observation has been confirmed by another more recent work by (Feliciano et al. 2017). Impairment of asynchronous GABA release and tonic inhibition results in an increased excitability at both single-neuron and network levels. An interesting observation is that, in SynI KO hippocampal brain slices, GABA release exhibits an opposite behavior. A decrease of synchronous eIPSC amplitude and an increase of asynchronous eIPSC amplitude has been observed (Forte et al. 2019). This opposite behavior of Syns I and II in regulating GABA release suggests the possibility that these proteins have non-redundant functions and constitute a sort of push–pull mechanism, regulating the ratio between synchronous and asynchronous release in inhibitory synapses in which they are co-expressed.

2.7. ROLE OF SYNAPSINS IN NEUROLOGICAL DISEASES

An interesting discovery is that mutations of human SYN genes are associated with severe diseases, such as epilepsy and autism spectrum disorder (ASD) (Garcia et al. 2004, Lakhan et al. 2010, Fassio et al. 2011, Corradi et al. 2014, Tang et al. 2015). Genetic and functional studies provide strong evidence that alteration in gene/protein expression are related to these disorders and this is an important step in the comprehension of synapsins' role in synaptic transmission regulation.

2.7.1. Epilepsy and autism spectrum disorders

Epilepsy is one of the most common neurological disorders; it affects about 1% of the total population. It is characterized by an abnormal and excessive neuronal activity caused by persistent cerebral dysfunction (Fisher et al. 2005). Genetic analyses in human populations have identified different mutations, especially in the SYN 1 and 2 genes, related to epilepsy and ASD etiology. Some of these mutations cause preferentially epilepsy or ASD, but some of them are at the base of both diseases. One of the first mutations that was discovered involves the SYN 1 gene. In this case, a nonsense mutation in the gene causes mRNA decay with a consequential lack of Syn I protein. The deficiency of the protein seems to be the cause of epilepsy in a family with history of epilepsy alone and is associated with aggressive behavior, learning disabilities or autism (Garcia et al. 2004). Another nonsense mutation in the SYN1 gene was identified in all affected individuals from a large French–Canadian family segregating epilepsy and autism spectrum disorders (ASDs) (Fassio et al. 2011). In addition, genetic mapping analysis identified variations in the SYN 2 gene as significantly contributing to epilepsy predisposition (Lakhan et al. 2010, Corradi et al. 2014, Prasad et al. 2014). The study of the synapsin KO mice model confirmed the

relationship between Syn genes and epilepsy. Despite the absence of gross defects in brain morphology, Syn I, Syn II, Syn I/II and Syn I/II/III (but not Syn III) KO mice exhibit early-onset spontaneous and sensory stimuli-evoked epileptic seizures. Spontaneous seizures develop after two months of age and progressively aggravate with aging and with the number of Syn genes ablated (Li et al. 1995, Rosahl et al. 1995). It is hard to explain the relationship between the molecular function of synapsins at the neuronal level and the onset of the epileptic phenotype. It seems that Syn mutations result in significant changes in synaptic transmission, plasticity and development. This could be potentially related to the appearance of an epileptic phenotype. Thus, the epileptic phenotype of Syn KO animals might be due to an imbalance in the activity of excitatory and inhibitory neurons (Baldelli et al. 2007, Chiappalone et al. 2009). In 2013, Lignani and collaborators conducted a study to understand the involvement of SynI mutations in the mechanisms of epileptogenesis. They suggested that the network hyperexcitability which leads to the manifestations of epilepsy/autism is triggered by imbalances in short term plasticity (STP) and the release dynamics of inhibitory and excitatory synapses (Lignani et al. 2013).

3. AIM OF THE STUDY

The aim of the project was to evaluate SynI contribution in the regulation of synaptic transmission at the presynaptic compartment. Synapsins present high affinity ATP binding domain; in particular, SynI binds ATP in a Ca^{2+}-dependent manner thanks to the coordination of a glutamate residue (E373). (Hosaka and Sudhof 1998). As ATP binding regulates SynI oligomerization and SV clustering (Orlando et al. 2014), we analyzed the effect of E373K mutation on neurotransmitter release and short-term plasticity in excitatory and inhibitory synapses. We coupled electrophysiology with electron microscopy in primary SynI KO hippocampal neurons in which either the human WT- or the E373K-SynI were re-introduced by infection with lentiviral vectors.

4. MATERIALS AND METHODS

4.1. PRIMARY AND AUTAPTIC CULTURES OF SYN I KO HIPPOCAMPAL NEURONS

Syn I KO mice were generated by homologous recombination. All experiments were carried out in accordance with the guidelines established by the European Community Council (Directive 2010/63/EU of September 22, 2010) and were approved by the Italian Ministry of Health. Pregnant females were killed by inhalation of CO_2, and embryonic day 17-18 (E17-18) embryos were removed immediately by cesarean section. Hippocampi were digested in 0.125% Trypsin for 15 min, then mechanically dissociated with a fire-polished Pasteur pipette and at the end plated on dot at low density (40 cells/mm^2). Autaptic neurons were prepared as described previously (Bekkers and Stevens 1991). Dissociated neurons were plated at very low density (20 cells/mm^2) on microdots (40-400 μm in diameter) obtained by spraying a mixture of poly-L-lysine (0.1 mg/ml) on dishes which had been pretreated with 0.15% agarose. All hippocampal culture were maintained in a culture medium consisting of Neurobasal, 2% B-27, 1% glutamax and 1% penicillin/streptomycin in a humidified 5% CO_2 atmosphere at 37 °C.

4.2. VIRUS PRODUCTION AND NEURON TRANSDUCTION

Sequences containing mCherry-WT-SynI and mCherry-E373K-SynI were cloned into pLenti6.2/V5-Dest plasmids (Invitrogen). The production of VSV-pseudo typed third-generation lentiviruses was performed as previously described (De Palma and Naldini 2002). Viral titers of about 1.0×10^9 TU/ml were obtained for both WT-SynI and E373K-SynI vectors. For all the experiments, hippocampal neurons were infected at 6 to 7 DIV at 10 multiplicity of infection. After 24 h from the infection, half of the

medium was replaced with fresh medium. The expression levels of WT- and E373K-SynI were assessed by mCherry fluorescence.

4.3. PATCH-CLAMP RECORDINGS FROM DISSOCIATED AND AUTAPTIC CULTURED HIPPOCAMPAL NEURONS

Hippocampal neurons were taken from Syn I KO mice and recorded at 12-15 DIV. Patch pipettes, prepared from thin borosilicate glass, were pulled and fire-polished to a final resistance of 4–5 MΩ when filled with standard internal solution. Voltage-clamp recordings were performed at a holding potential of −70 mV, and acquired at 10 to 20 kHz sample frequency. All experiments were performed at room temperature (22–24 °C). Data acquisition was performed using PatchMaster program (HEKA Elektronic). For all the experiments, cells were maintained in extracellular standard solution (Tyrode) containing (in mM): 140 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 4 KCl, 10 glucose, and 10 HEPES (pH 7.3 with NaOH). The internal solution (K-gluconate) was composed of (in mM) 126 K gluconate, 4 NaCl, 1 $MgSO_4$, 0.02 $CaCl_2$, 0.1 BAPTA, 15 glucose, 5 Hepes, 3 ATP, and 0.1 GTP (pH 7.3 with KOH). Evoked presynaptic currents (ePSCs) were recorded in Tyrode solution, for inhibitory transmission, containing adding D-AP5 (50 mM), CNQX (10 mM) and CGP58845 (5 mM) to block NMDA, non-NMDA and GABAB receptors, respectively. For excitatory transmission, D-AP5 (50 mM) and BIC (30 mM) were added to block NMDA and GABAA receptors, respectively. The size of the readily releasable pool (RRP) and the probability release (Pr) were calculated using the cumulative amplitude analysis during 2s tetanic stimulation at 40 Hz (Schneggenburger, Meyer et al. 1999). Data points in the linear range of the curves were fitted by linear regression and back-extrapolated to time 0. The intercept with the Y-axis gave the RRP and the

ratio between the amplitude of the first ePSC (I1) and RRP yielded the Pr. To study the response to paired-pulse protocols, we applied two consecutive stimuli at increasing interpulse intervals (20–1000 ms). Miniature postsynaptic currents (mPSCs) were recorded in voltage-clamp configuration in the presence of tetrodotoxin (TTX; 300 nM) in the extracellular solution to block the generation and propagation of spontaneous action potentials. The amplitude and frequency of mPSCs were calculated using a peak detector function using appropriate threshold amplitude and area. The frequency, amplitude and kinetics of miniature PSCs were analyzed using the MiniAnalysis program and the Prism software (GraphPad Software, Inc.). All the reagents were bought by Tocris, otherwise specified in details.

4.4. IMMUNOFLUORESCENCE

Primary hippocampal neurons were washed once in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. Cells were blocked with blocking buffer [5% goat serum (Vector Labs), 0.5% triton X-100] in PBS for 1 h at room temperature. Samples were sequentially incubated with primary antibodies overnight at 4 °C, followed by incubation with fluorochrome-conjugated secondary antibodies (Invitrogen) for 2 h at room temperature. After several washes in PBS, coverslips were mounted using Moviol reagent (Invitrogen). For quantification of the number of inhibitory and excitatory synapses, cultured neurons were stained for VGAT (1:500 dilution) and VGLUT1 (1:200 dilution). Neurons samples was visualized using a 63× objective in a fluorescence microscope (Leica). 18–35 z-stack for each image (step size 0.35 μm) were acquired. Image analyses were performed with ImageJ superimposing stacks of each color channel. The density of puncta was calculated as the number of vGLUT1-

or vGAT-positive puncta per 30 μm of proximal dendrite length. Manders coefficient of co-localization was calculated using the ImageJ JACoP plugin and expressed as the percentage of vGAT or vGLUT1 signal which overlaps with exogenous mCherry-SynI (WT or E373K) signal.

4.5. ELECTRON MICROSCOPY

Cultured hippocampal neurons derived from SynI KO embryos were infected at 7 DIV with either WT- and E373K-SynI and fixed at 14 DIV with 1.2% glutaraldehyde in 66 mM sodium cacodylate buffer, post-fixed in 1% OsO4, 1.5% K4Fe(CN)6, 0.1 M sodium cacodylate, en bloc stained with 1% uranyl acetate, dehydrated and flat-embedded in epoxy resin (Epon 812, TAAB). After baking for 48 h, the glass coverslips were removed from the Epon block by thermal shock and neurons were identified by means of a stereomicroscope. Embedded neurons were excised from the block, and mounted on a cured Epon block for sectioning using an EM UC6 ultramicrotome (Leica Microsystems). Ultrathin sections (60–70 nm thick) were collected on 200-mesh copper grids (Electron Microscopy Sciences) and observed with a JEM-1011 electron microscope (Jeol) operating at 100 kV using an ORIUS SC1000 CCD camera (Gatan, Pleasanton). For each experimental condition, at least 30 images of synapses were acquired at 10 000× magnification (sampled area per experimental condition: 36 μm^2). SV density and docked SV density were determined using ImageJ software. The analysis of synaptic ultrastructure at the end of the train stimulation protocol (30 s at 10/20 Hz) or after recovery (150 s at 0.1 Hz) was performed by fixing the samples with glutaraldehyde at 37° C. Under these conditions, we estimated a complete fixation of synapses within 1–3 s from fixative addition (Leung, Brennan et al. 1994).

4.6. MEA RECORDINGS

Dissociated hippocampal neurons were plated onto a planar Muse MultiElectrode Array (Axion Biosystems, Atlanta, GA, USA). The electrode diameter was 30 µm, and the orthogonal distances between electrodes were 200 µm. The day before dissociation, the active electrode area was coated overnight with poly-L-lysine (0.1 mg/ml). The Muse 64 channel amplifier (gain 1200, 61 dB) connected to an external hardware controller via a National Instrument analog-to-digital card was used to amplify extracellular raw data. Raw data were digitized at 20 kHz and stored on a hard disk for off-line analysis. Spike detection of single extracellular action potentials was performed using the Axion Biosystem software using a voltage threshold of six times the standard deviation of the noise over 200 Hz high-pass filtered traces. Spike train data were analyzed using the Neuroexplorer software (Plexon, Dallas, TX, USA). Bursts were detected using the burst analysis algorithm of Neuroexplorer with the following criteria: maximum inter-spike interval 200 ms, minimum burst duration 20 ms and minimum number of spikes per burst five. Cultures were recorded at 14 and 21 DIV under control conditions (complete Neurobasal) in the MEA chamber maintained at 37 °C for at least 30 min.

4.7. STATISTICAL ANALYSIS

Data were analyzed by Student's t-test or, in case of more than two experimental groups, by one-way ANOVA followed by post-hoc multiple comparison tests. If the data were not normally distributed, nonparametric statistical tests (Mann-Whitney test or the Kruskal-Wallis multiple comparison tests) were used. Significance level was preset to $p < 0.05$ for all tests. Data are expressed as medians ± SEM, for number of cells.

The normal distribution of experimental data was assessed using the Kolmogorov-Smirnov test. Statistical analysis was carried out by using Prism software (GraphPad Software, Inc.).

5. RESULTS

5.1. E373K-SynI increases miniature excitatory postsynaptic currents frequency.

We investigated whether the expression of E373K-SynI could alter the physiological properties of either excitatory and inhibitory miniature postsynaptic currents (mPSCs). No changes in miniature EPSCs (mEPSCs) and miniature IPSCs (mIPSCs) amplitude were observed in synapses expressing either WT- or E373K-SynI, thus excluding postsynaptic effects. However, whereas mIPSC frequency was not affected, mEPSC frequency was significantly increased in E373K-SynI synapses (Fig. 1A-D). Enhancement of miniature event frequency is normally attributable to two distinct factors: (i) an increased number of synapses or (ii) an increased probability of spontaneous SV fusion. To test the first possibility, we evaluated synaptic density by co-immunostaining hippocampal neurons with two presynaptic markers: vGLUT1 and vGAT for excitatory and inhibitory synapses, respectively.

The analysis of the Manders' overlap coefficient revealed that both mCherry-positive puncta of WT- and E373K-SynI similarly co-localize with vGAT- and vGLUT1-positive puncta, that identify putative inhibitory and excitatory synaptic contacts. The analysis demonstrated that about 50% of both WT- and E373K-SynI was localized at the presynaptic boutons (Fig. 2B and C, upper panels).

Moreover, no difference was observed in synaptic density between genotypes (Fig. 2A-C). These data indicate that mutated SynI does not affect the balance between excitatory and inhibitory synaptic contacts suggesting that the enhancement of mEPSCs frequency could be due to an alteration of presynaptic release machinery.

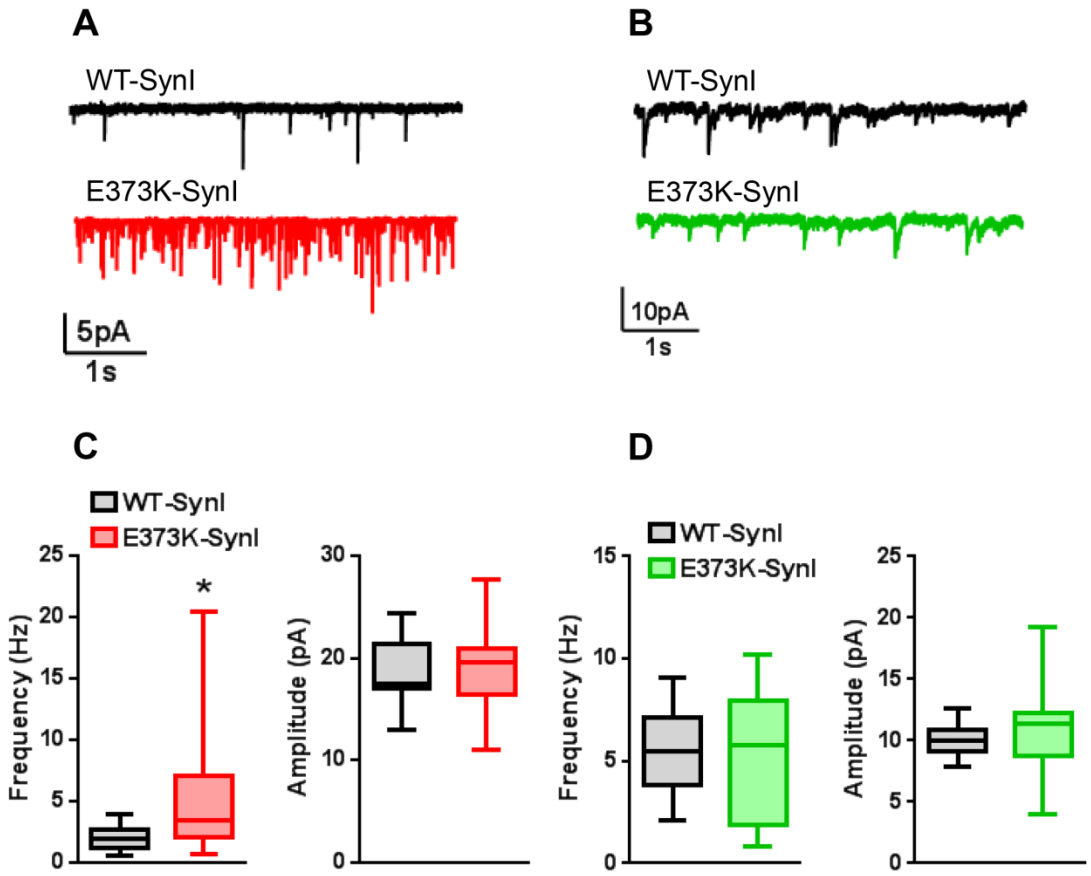


Figure 1. Expression of mutant E373K-SynI increases mEPSCs frequency. (A-B) Representative traces of excitatory **(A)** and inhibitory **(B)** miniature postsynaptic currents recorded at -70 mV in 14 DIV SynI KO hippocampal neurons transduced with either WT-SynI (black) and E373K-SynI (color). **(C-D)** mEPSC **(C)** and mIPSC **(D)** frequency and amplitude plots. All data are medians ± SEM from the indicated numbers of cells recorded from n=3 independent cell culture preparations. Excitatory synapses: n=20 and n=20; inhibitory synapses: n=12 and n=14; for WT- and E373K-SynI, respectively. *p<0.05; Mann-Whitney U test.

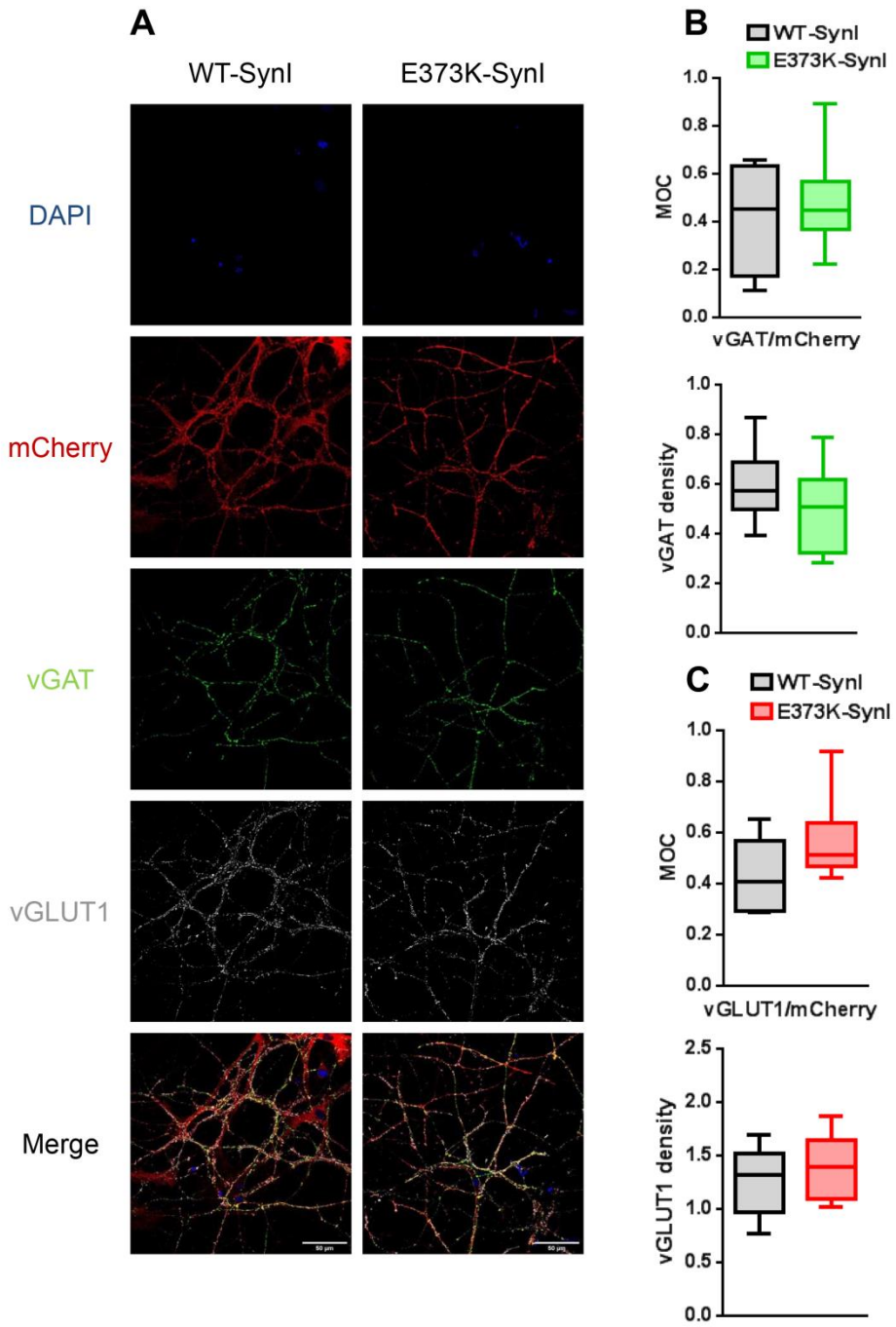


Figure 2. Mutant SynI does not influence either excitatory or inhibitory synaptic density. (A) Representative images of WT- and E373K-SynI transduced hippocampal neurons at 14 DIV triple stained for mCherry (red), vGLUT1 (gray) and vGAT (green). Nuclei were stained with DAPI. Scale bar, 50 µm. **(B)** Manders' overlap coefficient of vGAT- and mCherry-positive inhibitory synapses (top) and vGAT density (bottom) **(C)** Mander's overlap coefficient of vGLUT1- and mCherry-positive excitatory synapses (top) and vGLUT1 density (bottom). All data are medians ± SEM from the indicated numbers of cells recorded from n=2 independent cell culture preparations. $p > 0.05$, Mann-Whitney U test.

5.2. Presynaptic calcium chelation restores completely miniature excitatory postsynaptic currents frequency.

Since synaptic density was not altered in E373K-SynI neurons, we investigated the possible functional alteration of the presynaptic release machinery. It is known that, in the absence of an increased number of synapses, enhancement in mPSC frequency can be due to two factors: (i) the increase of Ca^{2+} oscillations or/and (ii) an increase in the number of docked SVs. To test the first hypothesis, we recorded mPSC events before and after the treatment with BAPTA-AM, an efficient and fast Ca^{2+} chelator. Both excitatory and inhibitory mPSCs were initially recorded in control extracellular solution for 2 min. After the addition of BAPTA-AM (2 μM) to the external solution, mPSCs were recorded for further 15 min and analyzed in three consecutive time windows of 5 min (Fig 3A-C). Inhibitory mPSC frequency were similarly not affected by BAPTA-AM treatment in both genotypes (Fig. 3A-C). On the contrary, the increased mEPSC frequency that characterized SynI KO neurons transduced with E373K-SynI was totally suppressed by BAPTA-AM treatment (Fig. 4A-B). On the contrary, mEPSC amplitude resulted similarly unchanged by BAPTA-AM, in both genotypes (Fig. 4C). Thus, these data suggest that the enhancement of mEPSC frequency could be due to an alteration in presynaptic Ca^{2+} homeostasis. Results were obtained from pure embryonic neuronal primary cultures with only occasional presence of astrocytes. This allows us to exclude that the mEPSC frequency reduction was due to the indirect action of BATPA-AM on astroglial Ca^{2+} waves.

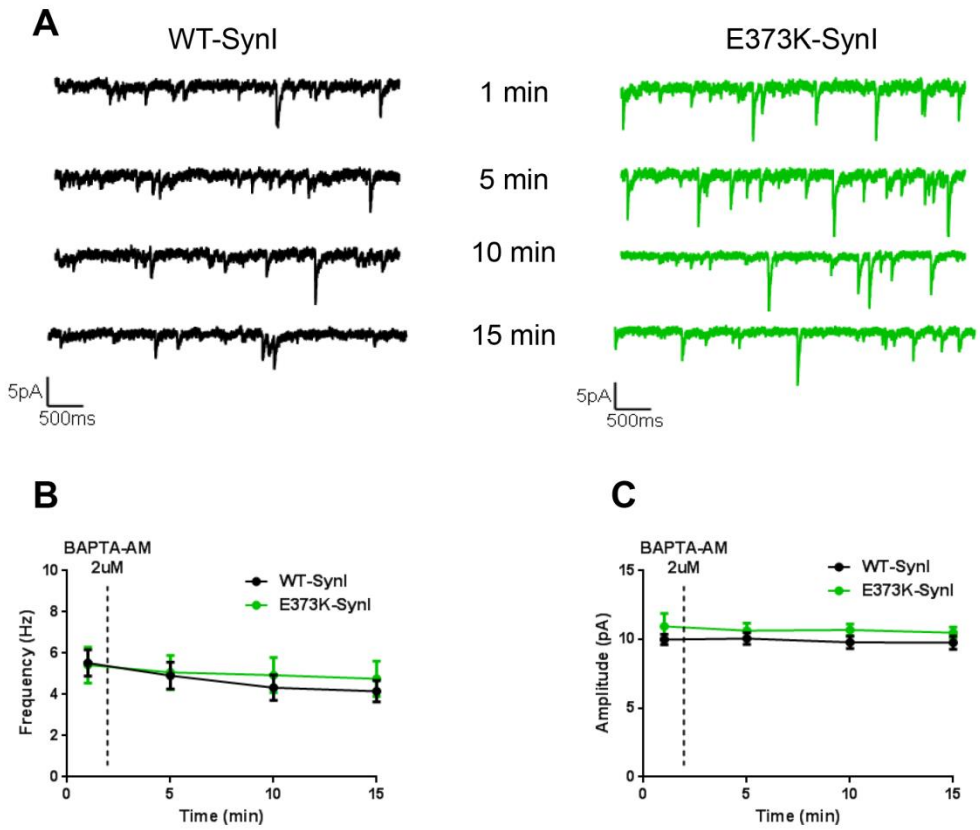


Figure 3. Expression of mutant E373K-SynI does not alter mIPSCs before and after BAPTA-AM extracellular injection. (A) Representative traces of miniature inhibitory postsynaptic currents recorded at -70 mV in 14 DIV hippocampal neurons transduced with WT-SynI (left, black, n=15) and E373K-SynI (right, green, n=14) at 4 time points (1, 5, 10 and 15 min). **(B)** Frequency and **(C)** amplitude plots. Data are medians ± SEM from the indicated numbers of cells recorded from n=3 independent cell culture preparations. One-way ANOVA Bonferroni's multiple comparisons test.

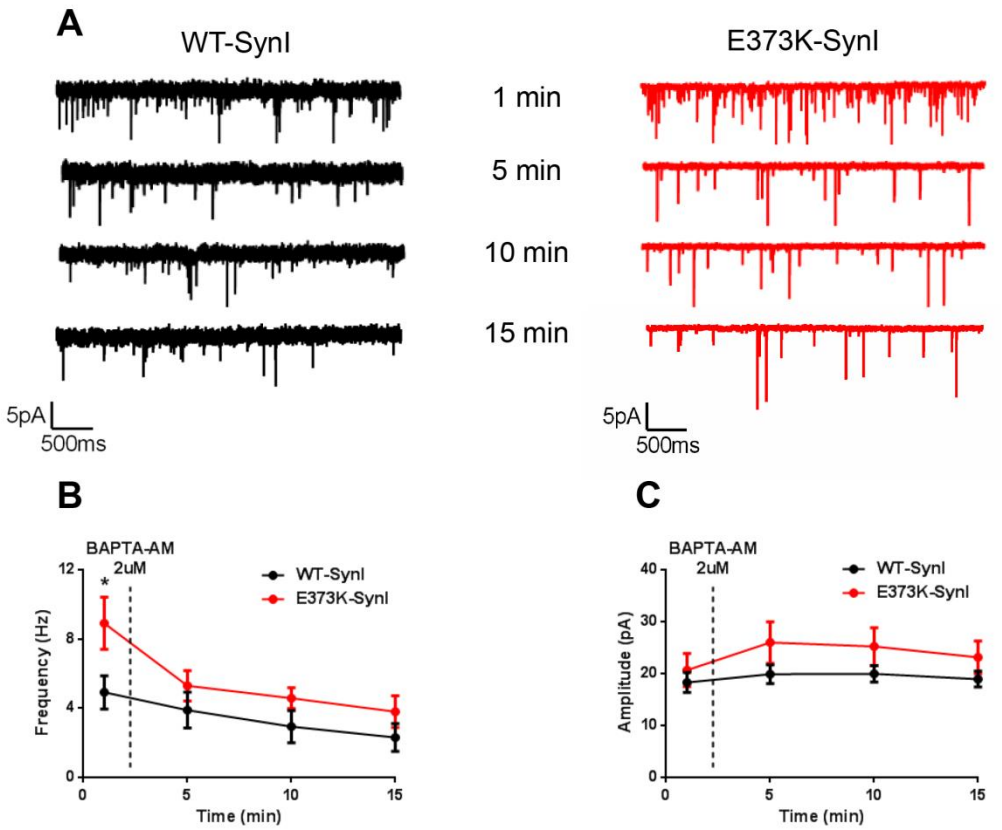


Figure 4. The enhancement of mEPSC frequency induced by E373K-SynI is suppressed by BAPTA-AM treatment. (A) Representative traces of miniature excitatory postsynaptic currents recorded at -70 mV in 14 DIV hippocampal neurons transduced with WT-SynI (left, black, n=6) and E373K-SynI (right, red, n=8) at 4 time points (1, 5, 10 and 15 min). **(B)** Frequency and **(C)** amplitude plots. Data are medians ± SEM from the indicated numbers of cells recorded from n=3 independent cell culture preparations. *p<0.05; One-way ANOVA Bonferroni's multiple comparisons test.

5.3. The mutated E373K-SynI reduces eEPSCs, but not eIPSCs.

In order to investigate in detail, the mechanism underlying the synaptic functional changes induced by E373K-SynI, we studied evoked (e) excitatory and inhibitory PSCs. It was extensively reported that SynI plays a crucial role in the storage and the mobilization of SVs, regulating synaptic transmission (Cesca et al. 2010).

Here, we investigated ePSCs and various forms of STP of both excitatory and inhibitory synapses expressing native- or mutated-E373K SynI, using: paired pulse-stimulation, short- and long-lasting high frequency stimulation (HFS). To evaluate STP, we generated two different cell cultures: a low density neuronal culture in which the presynaptic neurons were stimulated with an external stimulator for all inhibitory synaptic transmission experiments; and an autaptic neuronal culture, in which we applied a stimulation and recorded the evoked current in the very same neuron for all excitatory synaptic transmission experiments (Fig. 5A). External stimulation was performed by positioning the stimulator near the putative presynaptic neuron and injecting current in a range between 200 to 500 µA. This range of current was sufficient to generate a postsynaptic response, recruiting approximately only one presynaptic fiber. In autaptic neurons, evoked current was generated by depolarizing cell membrane potential to +40 mV.

Single pulse stimulation revealed that excitatory synapses expressing the mutated E373K-SynI showed a significant reduction in evoked amplitude (Fig. 5C); on the contrary, no changes were observed in inhibitory synapses transduced with E373K-SynI (Fig. 5B). The paired-pulse stimulation is based on two consecutive stimuli applied at inter-pulse intervals ranging from 25 ms to 1 s. The two stimuli generated two different postsynaptic currents. After measuring the amplitude of the two

postsynaptic events, we calculated the ratio between the second (I2) and the first one (I1), obtaining the paired pulse ratio (I2/I1) (Fig. 5D-E). At short intervals (25–100 ms), both excitatory and inhibitory synapses expressing E373K-SynI displayed no difference in paired-pulse ratio compared to WT-SynI expressing synapses (Fig. 5F-G). Notably, with an inter-pulse interval of 200 ms, inhibitory synapses expressing the mutated SynI showed a reduced paired pulse ratio (Fig. 5F).

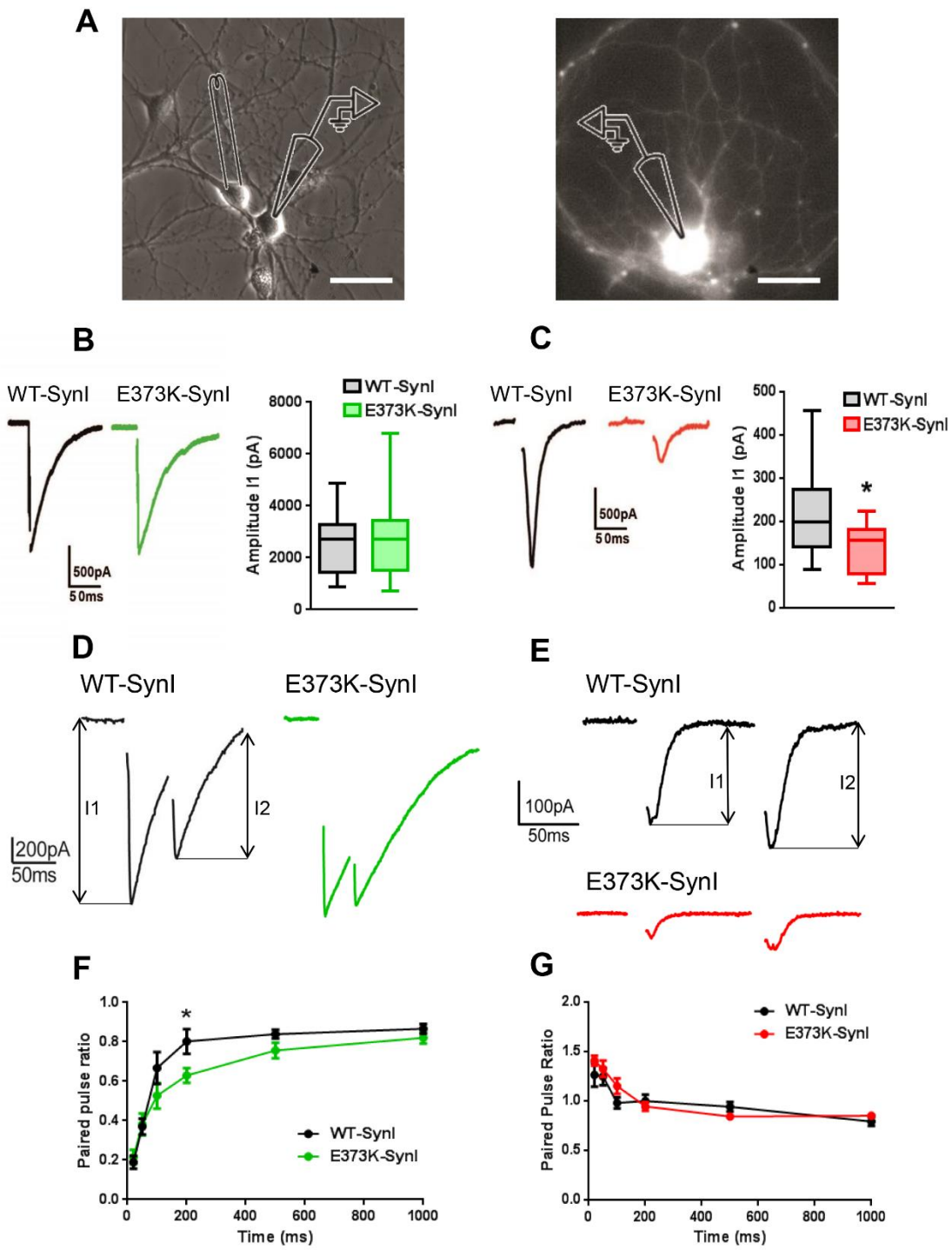

A
B
WT-SynI
E373K-SynI
500pA
50ms
Amplitude I1 (pA)
8000
6000
4000
2000
0
C
WT-SynI
E373K-SynI
500pA
50ms
Amplitude I1 (pA)
500
400
300
200
100
0
*
D
WT-SynI
E373K-SynI
I1
I2
200pA
50ms
E
WT-SynI
I1
I2
100pA
50ms
E373K-SynI
F
Paired pulse ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
WT-SynI
E373K-SynI
0
200
400
600
800
1000
Time (ms)
G
Paired Pulse Ratio
2.0
1.5
1.0
0.5
0.0
WT-SynI
E373K-SynI
0
200
400
600
800
1000
Time (ms)

Figure 5. Mutant E373K-SynI induces a significant reduction of eEPSC amplitude without affecting eIPSC. (A) Representative images of low density neuronal cultures (left, scale bar, 50 µm) and autaptic neurons (right, scale bar, 15 µm). **(B-C)** Single pulse protocols were applied to inhibitory **(B, left)** and excitatory **(C, left)** SynI KO synapses transduced with either WT-SynI (black) or E373K-SynI (color). Histograms indicating the amplitude of eIPSCs **(B, right)** and eEPSCs **(C, right)**. **(D-E)** Paired-pulse protocols were applied to inhibitory **(D)** and excitatory **(E)** SynI KO synapses transduced with either WT-SynI (black) or E373K-SynI (color). **(F-G)** Paired-pulse ratio of inhibitory **(F)** and excitatory **(G)** synapses transduced with either WT-SynI (black) or E373K-SynI (color) at interpulse intervals ranging from 25 ms to 1 s. All data are medians ± SEM from the indicated numbers of cells recorded from n=3 independent cell culture preparations. Inhibitory synapses: n=13 and n=15; excitatory synapses: n=19 and n=14; for WT- and E373K-SynI, respectively. *$p<0.05$; Mann-Whitney U test.

5.4. E373K-SynI alters STP specifically at excitatory synapses.

With the aim of defining to what extent mutated SynI modulates the quantal parameters of synchronous glutamate and GABA release, we estimated the readily releasable pool for synchronous release (RRPsyn) and the probability of release of any given SV in the RRP (Pr) using cumulative amplitude analysis.

Both excitatory and inhibitory synapses were stimulated at 40 Hz for 1 s and cumulative amplitude was obtained (Fig. 6A-B). When neurons were challenged with the train a significant depression of ePSCs became apparent during the stimulation period irrespective of the amplitude of the first current in the train. Accordingly, the cumulative profile of the ePSC amplitude displayed a rapid rise followed by a slower linear increase reflecting the equilibrium between depletion and constant replenishment of the RRP. The cumulative amplitude profiles of the data points between 0.5 and 1 s were fitted by linear regression and back extrapolated to time 0 to obtain the RRP_{syn} (Fig. 6C-D). The Pr was obtained from the ratio between the amplitude of the first event and the $RRP_{syn.}$ No changes were observed in inhibitory synapses in terms of amplitude, RRP_{syn} and Pr (Fig. 6E-G). Interestingly, SynI KO excitatory neurons transduced with E373K-SynI showed a significantly reduction of both amplitude and RRP_{syn}, with no change in Pr (Fig. 6F-H).

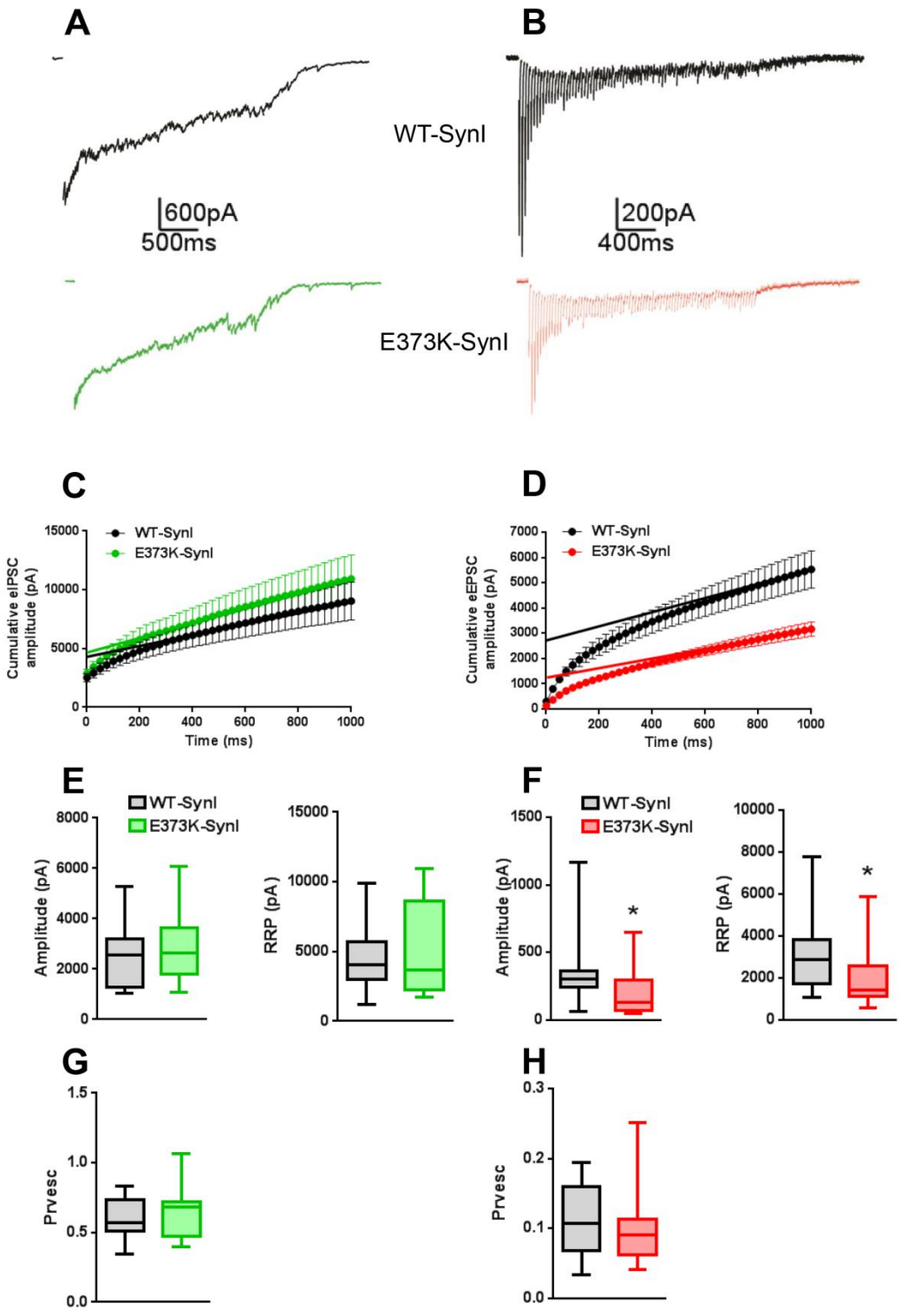

A
B
WT-SynI
600pA
500ms
200pA
400ms
E373K-SynI
C
D
WT-SynI
E373K-SynI
Cumulative eIPSC amplitude (pA)
Cumulative eEPSC amplitude (pA)
Time (ms)
E
F
Amplitude (pA)
RRP (pA)
*
G
H
Prvesc

Figure 6. Mutant E373K-SynI decreases synchronous release in excitatory synapses without affecting inhibitory ones. (A–D) Representative traces showing the stimulation protocol of a 1 s at 40 Hz, used to estimate the quantal properties of synchronous release in inhibitory **(A)** and excitatory **(B)** SynI KO synapses transduced with either WT-SynI (black) or E373K-SynI (color). **(C-D)** The cumulative curves of eIPSCs **(C)** and eEPSCs **(D)** amplitude are shown. **(E-H)** Histograms of the amplitude of the first peak, the readily releasable pool (RRP_{syn}) for synchronous release and the release probability (Pr) are shown for inhibitory **(E and G)** and excitatory **(F and H)** synapses. All data are medians ± SEM from the indicated numbers of cells recorded from n=3 independent cell culture preparations. Inhibitory synapses: n=13 and n=15; excitatory synapses: n=19 and n=19; for W- and E373K-SynI, respectively. *$p<0.05$; Mann-Whitney U test.

5.5. E373K mutation affects the recovery from synaptic depression induced by sustained HFS in both excitatory and inhibitory synapses.

It was previously reported that deletion of Syns enhances synaptic depression during sustained high frequency stimulation (HFS), although the effect appears to be more intense at excitatory than at inhibitory synapses (Gitler, Xu et al. 2004, Baldelli, Fassio et al. 2007). Excitatory and inhibitory synapses of neurons transduced with both WT- and E373K-SynI were subjected to 30 s HFS (20 and 10 Hz, respectively). The progressive decay of ePSCs amplitude during the train and the subsequent recovery from depression (Fig. 7A and 8A) was analyzed and compared in excitatory and inhibitory synapses expressing WT- or mutated-Syn I. During HFS, E373K-SynI inhibitory synapses showed no significant difference in slow and fast time constants of depression, but showed a lower steady-state current (SSC) (Fig. 7B-E). In addition, mutated inhibitory synapses also failed to fully recover after the train with no difference in the dynamic (Fig. 7F-G). During HFS, E373K-SynI excitatory synapses presented no difference in the SSC and the depression dynamics (Fig. 8B-E) but, similarly to inhibitory synapses, showed an impairment in the recovery after depression (Fig. 8F-G). This result suggests that E373K mutation impairs SVs recruitment and recycling in response to sustained HFS in both excitatory and inhibitory synapses.

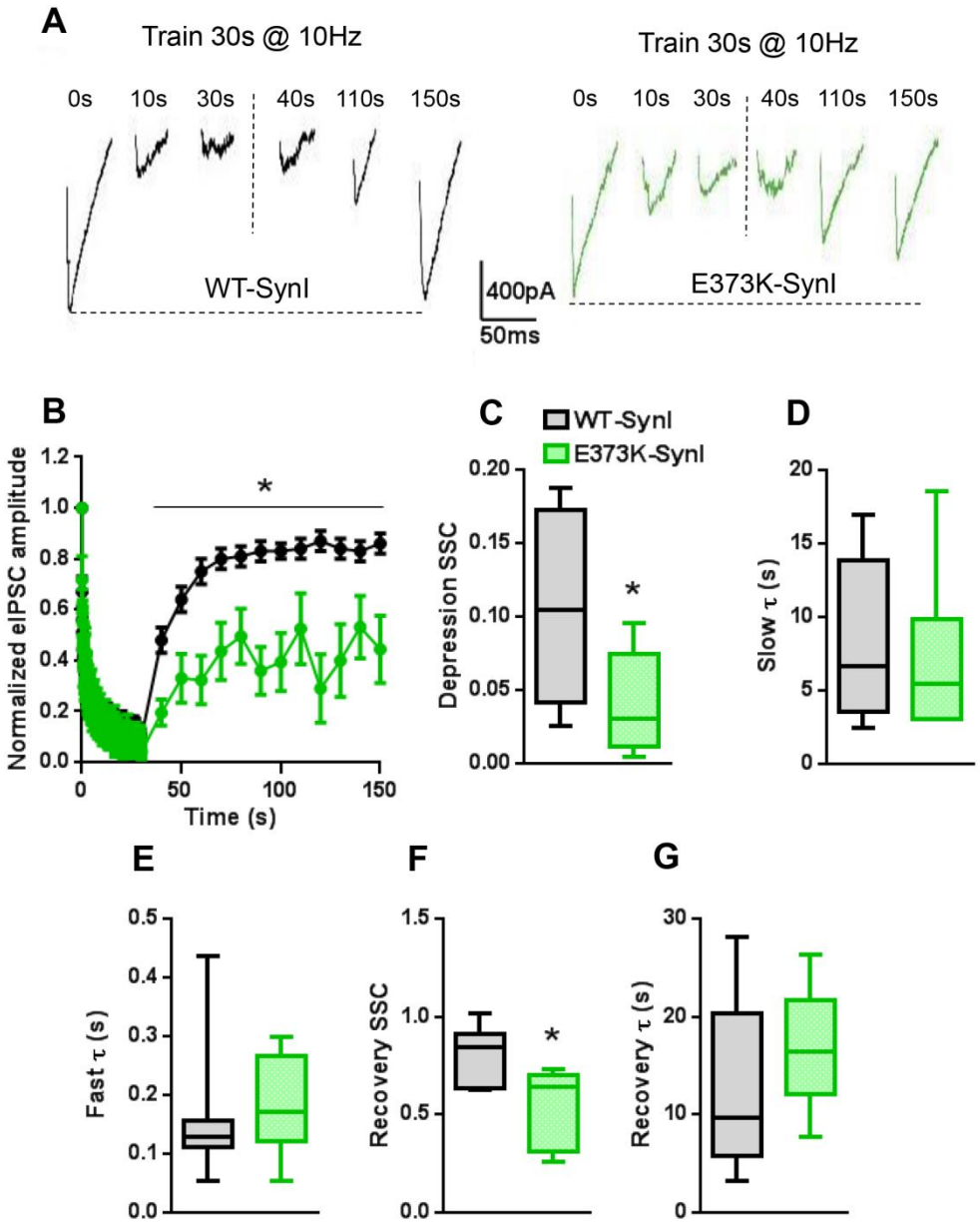


Figure 7. Mutant SynI increases depression during sustained high frequency stimulation (HFS) and fails to recover eIPSC in inhibitory synapses. **(A)** Representative traces of synaptic depression evoked by stimulating inhibitory SynI KO neurons transduced with either WT-SynI (black; n=11) and E373K-SynI (green; n=6) with trains lasting 30 s at 10 Hz. **(B)** The progressive decay of eIPSC amplitude during the stimulation train and the subsequent recovery from the depression are plotted as a function of time from the beginning of the train. **(C-E)** Depression steady state current **(C)**, slow **(D)** and fast **(E)** τ are shown in the box plots. **(F-G)** Recovery steady state **(F)** and τ of recovery **(G)** are shown in the box plots. All data are medians ± SEM from the indicated numbers of cells recorded from n=3 independent cell culture preparations. *p< 0.05; Mann-Whitney U test.

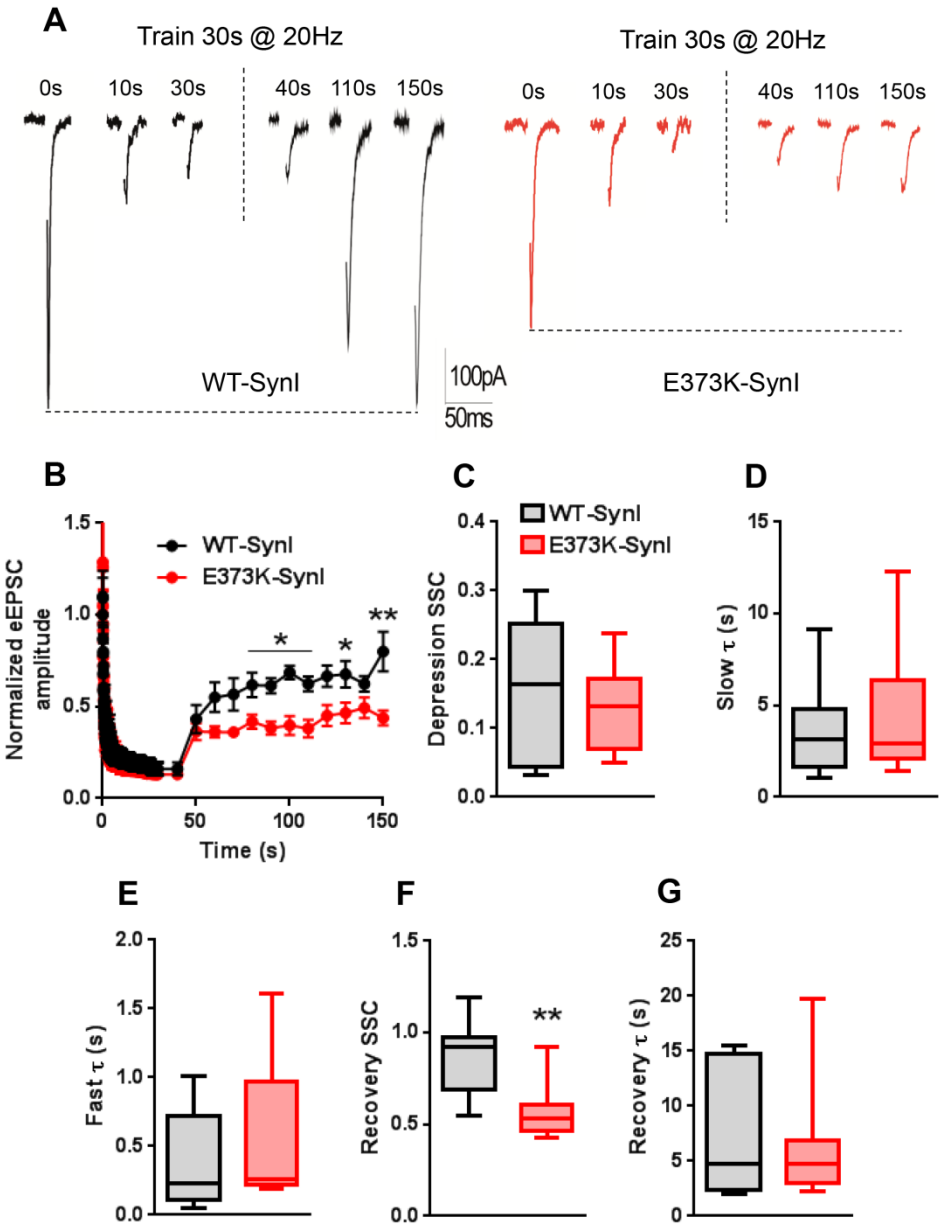


Figure 8. Mutant SynI fails to recover eEPSC after sustained high frequency stimulation (HFS) in excitatory synapses. **(A)** Representative traces of synaptic depression evoked by stimulating autaptic excitatory SynI KO neurons transduced with either WT-SynI (black, n=9) and E373k-SynI (red, n=8) with trains lasting 30 s at 20 Hz. **(B)** The progressive decay of eEPSCs amplitude during the stimulation train, and the subsequent recovery from depression are plotted as a function of time from the beginning of the train. **(C-E).** Depression steady state current **(C)**, slow **(D)** and fast **(E)** τ are shown in the box plots. **(F-G)** Recovery steady state **(F)** and τ of recovery **(G)** are shown in the box plots. All data are medians ± SEM from the indicated numbers of cells recorded from n=3 independent cell culture preparations. *p< 0.05; **p<0.01; Mann-Whitney U test.

5.6. E373K-SynI affects synaptic vesicle trafficking after recovery in both excitatory and inhibitory synapses.

To verify if the defect in recovery from synaptic depression was due to an alteration in the SV trafficking, we stimulated SynI KO hippocampal neurons transduced with both WT- and E373K-SynI with the same protocol adopted for electrophysiological experiments and fixed the samples for electron microscopy. We evaluated total SV density of the presynaptic terminal and docked SV density in three different conditions: (i) *pre-HFS* (30 s at 20 Hz); 1 s before the start of HFS, (ii) *end-HFS*; 1 s after the end of HFS and (iii) *after-HFS*; 150 s after the end of the HFS (Fig 9A and 10A). Transmission electron microscopy followed by ultrastructural analysis revealed that E373K-SynI inhibitory terminals were relatively similar compared to WT-SynI synapses in terms of total and docked SV densities in *pre-HFS* and *end-HFS* conditions, but presented a significant depletion of the total SV density 150 s after the end of the HFS (*after-HFS*) (Fig. 9B). Interestingly, excitatory synapses transduced with mutant SynI showed a significant reduction of total SV density under basal condition (*pre-HFS*). This effect was lost in the condition of deep synaptic depression reached at the end of the HFS (*end-HFS*) but, similarly with inhibitory synapses, excitatory synapses failed to fully recover the basal SV density, 150 s after the end of the HFS (*after-HFS*) (Fig. 10B). Notably, the number of docked SVs never changed under all conditions for both types of synapses and genotypes. Taken together, these results show that in both excitatory and inhibitory synapses the absence of Ca^{2+} binding to SynI, dysregulates the SynI-mediated trafficking of SVs in terminals challenged with sustained HFS. Moreover, only excitatory synapses expressing mutant SynI failed to maintain a correct distribution of SVs under basal conditions.

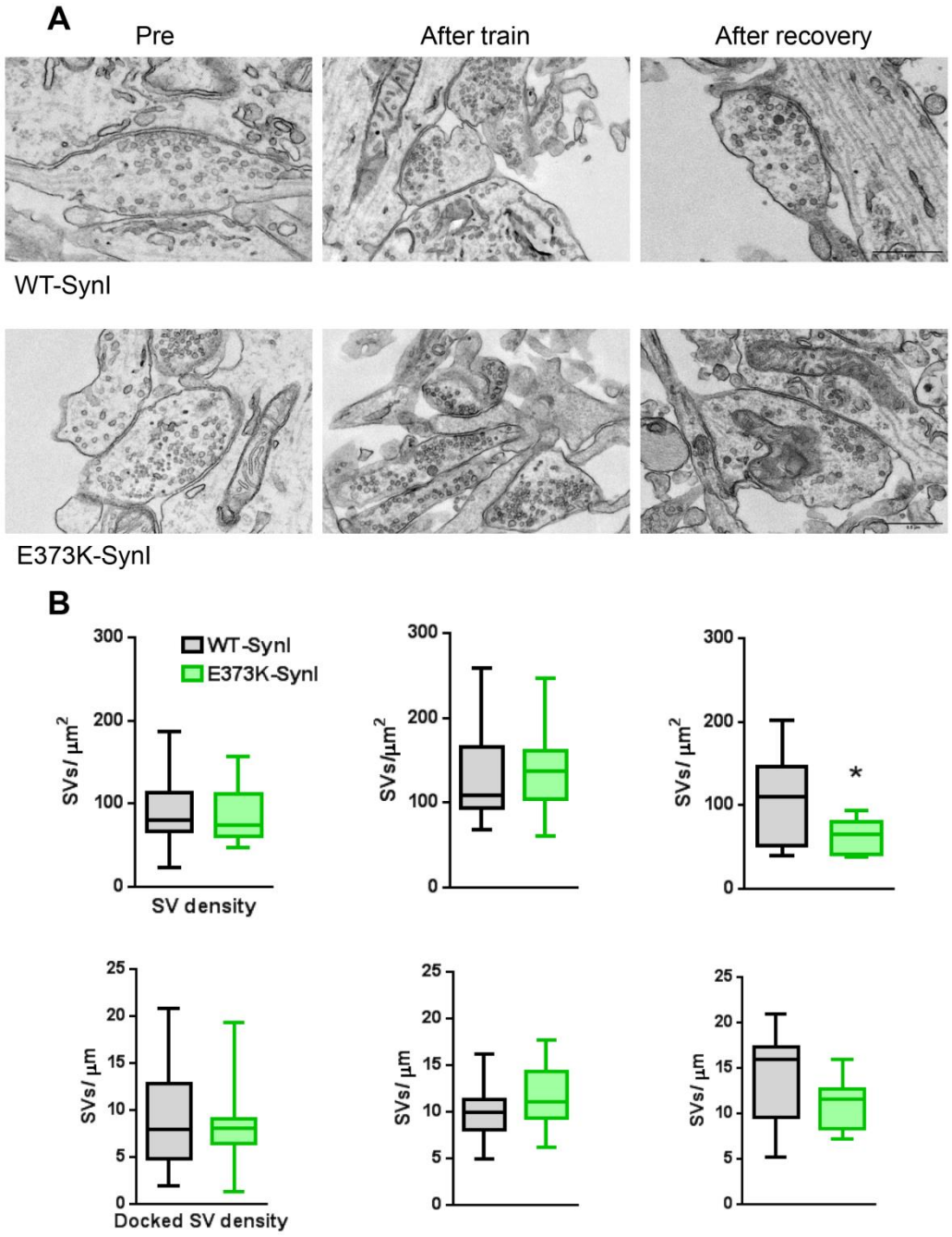


Figure 9. The total SV density of mutant SynI inhibitory neurons fails to recover its basal value after a sustained HFS. (A) Representative transmission electron micrographs of inhibitory presynaptic terminals from SynI KO neurons transduced with either WT- and E373K-SynI (top and bottom rows; n=15 and n=8, respectively). Synaptic ultrastructure was evaluated by fixing neurons under basal conditions, after the train at 10 Hz for 30 s and after 150 s of recovery (left, meddle and right columns, respectively). Scale bar: 500 nm. **(B)** The total SV density (top row) and the number of docked SVs (bottom row) are plotted. All data are medians ± SEM from the indicated numbers of cells recorded from n=3 independent cell culture preparations. *p< 0.05; Mann-Whitney U test.

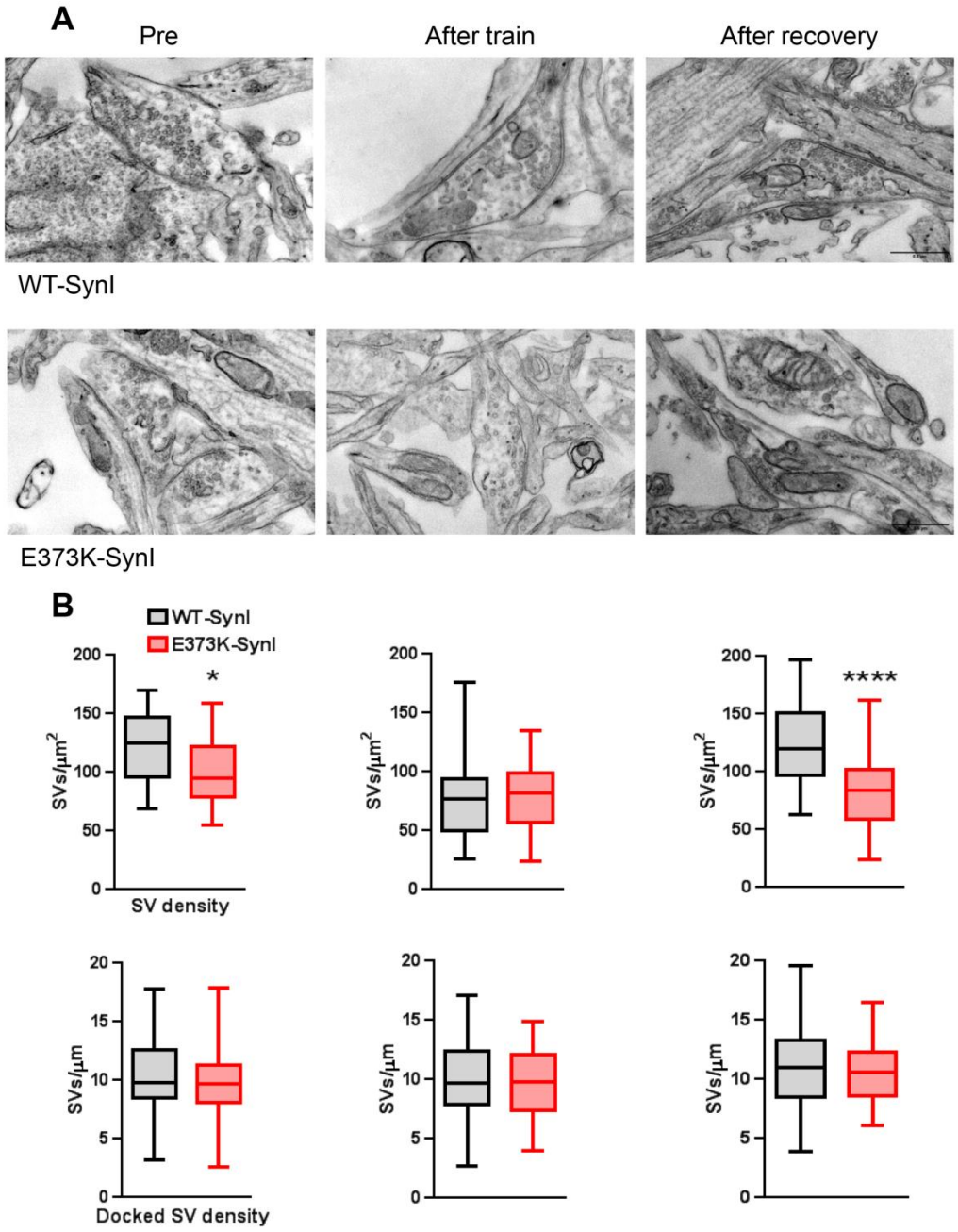


Figure 10. Excitatory synapses expressing mutant SynI showed a reduced total SV density that is unable to recover its control value at the end of a sustained HPF. (A) Representative transmission electron micrographs of excitatory presynaptic terminals from SynI KO neurons transduced with either WT- and E373K-SynI (top and bottom rows, respectively; n=30). Synaptic ultrastructure was evaluated by fixing neurons under basal conditions, after the train at 20 Hz for 30 s and after 150 s of recovery (left, meddle and right columns, respectively). Scale bar: 500 nm. **(B)** The total SV density (top row) and the number of docked SVs (bottom row) are plotted. All data are medians ± SEM from the indicated numbers of cells recorded from n=3 independent cell culture preparations. *p< 0.05; **** p<0.0001; Mann-Whitney U test.

5.7. The excitatory/inhibitory alterations in STP do not affect network excitability in control conditions.

In order to study the consequences of changes in STP properties induced by mutant-SynI at the network level, we used MEA recordings of the reverberating spontaneous electrical activity of cultured hippocampal neurons (14-21 DIV) (Fig. 11A). Spontaneous network activity was investigated in terms of both sparse isolated spikes and organized high frequency bursts (Fig. 11B-E) that could last up to 100 ms (van Pelt et al. 2004, Chiappalone et al. 2007, Vajda et al. 2008). Under control conditions, mutant SynI transduced networks exhibited no difference in terms of firing rate, burst duration and frequency at both 14 and 21 DIV (Fig. 11B-E). These results suggest that the impairment in excitatory transmission, coupled with the deficiency in recovery after sustained HFS and the alteration in SV density of both inhibitory and excitatory synapses, do not affect significantly neuronal network excitability.

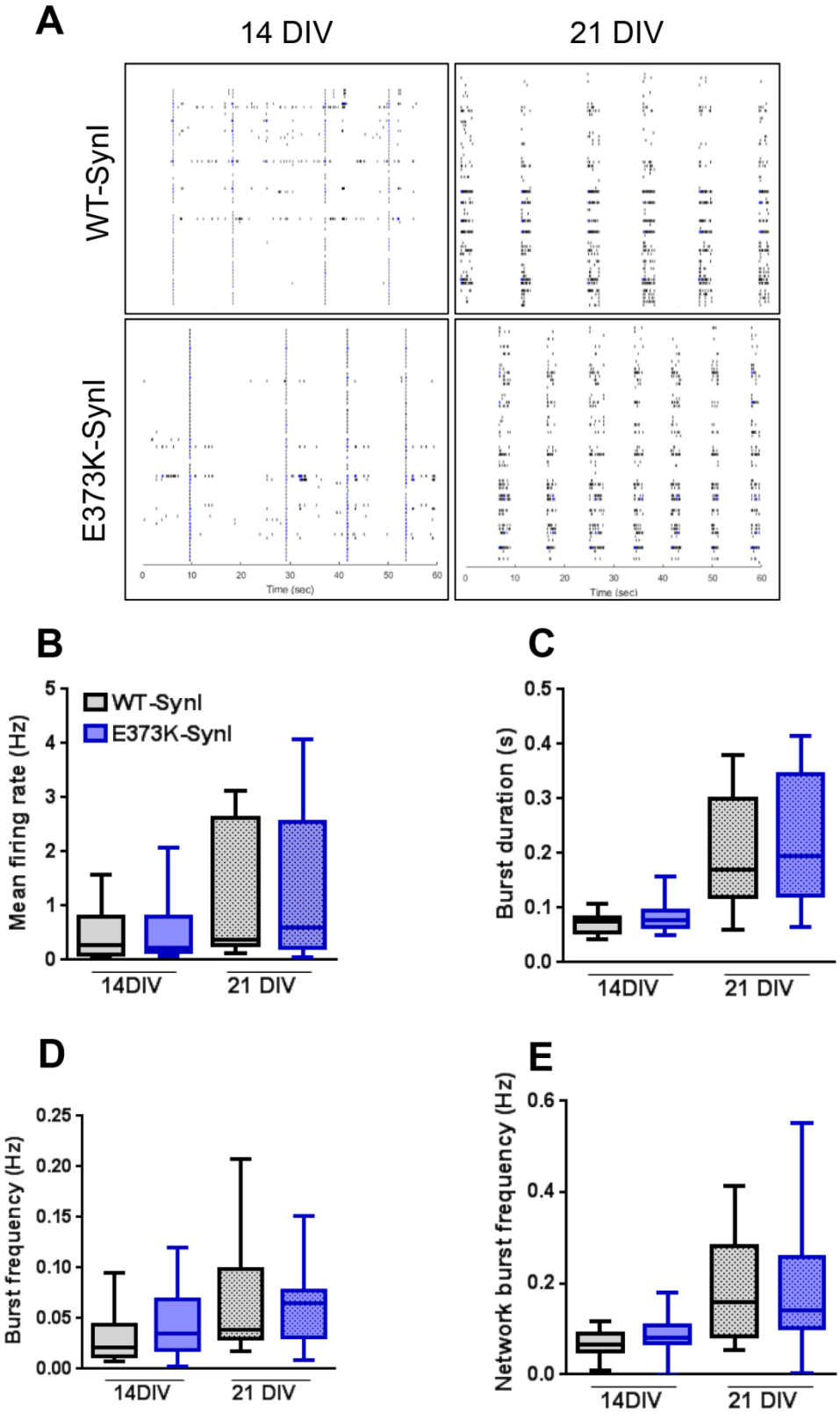

A
14 DIV
21 DIV
WT-Synl
E373K-Synl
Time (sec)
B
Mean firing rate (Hz)
WT-Synl
E373K-Synl
14DIV
21 DIV
C
Burst duration (s)
14DIV
21 DIV
D
Burst frequency (Hz)
14DIV
21 DIV
E
Network burst frequency (Hz)
14DIV
21 DIV

Figure 11. The expression of mutant SynI does not affect the spontaneous firing activity of hippocampal neuronal networks. (A) Representative raster plots of spontaneous activity at 14 DIV (left) and 21 DIV (right) showing spiking and bursting activities. **(B–E)** Mean firing frequency **(B)**, burst duration **(C)**, burst frequency **(D)** and network burst frequency **(E)** measured from the raster plots of spontaneous activity, are plotted for WT-SynI (black) or E373K-SynI (blue) networks. All data are medians ± SEM from the indicated numbers of cells recorded at least from n=3 independent cell culture preparations. Neurons at 14 DIV: n=35 and n=35; neurons at 21 DIV: n=26 and n=26, for WT- and E373K-SynI, respectively.

6. DISCUSSION

For decades, it has been demonstrated that in neurons Ca^{2+} plays a key role in synaptic transmission dynamics. When an action potential invades the nerve terminal, it causes the opening of VGCCs with a transient increase of Ca^{2+} intracellular concentration, particularly at the active zone. Ca^{2+}, acting as second messenger, triggers SV fusion and the subsequent neurotransmitter release. Katz and Miledi hypothesized this fundamental mechanism for the first time in 1967 while studying the neuromuscular junction. At the same time, several studies were introduced that utilized different models, such as giant squid axon synapses (Augustine et al. 1985) and rat cerebellar parallel fiber synapses (Sabatini and Regehr 1996). More precise studies that elucidated this process, have been performed using the calyx of Held synapses as model (Brew and Forsythe 1995, Sakaba and Neher 2001, Meinrenken et al. 2002). Despite the large amount of studies published, the specific mechanism that links Ca^{2+} influx and SV fusion has been elucidated by Schiavo and collaborators in 1992. They hypothesized that synaptobrevin-2, a SV protein, formed a complex with two proteins of the neuronal plasma membrane (syntaxin and SNAP25), triggering SV fusion (Schiavo et al. 1992). But, the role of Ca^{2+} is not limited to the fusion process, as it has been demonstrated that its presence is required for the recruitment of SVs, particularly through the interaction with synapsins. First in 1998, Esser and collaborators obtained a crystal structure of the C-domain of bovine SynI. They demonstrated that this domain shares homology with "ATP-grasp" proteins and contains a flexible loop (MFL) able to bind ATP (Esser et al., 1998). The fact that SynI also presents a Ca^{2+}-binding domain (through a glutamate residue, Glu373) led to the hypothesis that ATP binding is regulated by Ca^{2+}. Indeed, in 1998, Hosaka and Sudhof suggested that ablation of Glu373 and its substitution with a lysine residue

converts SynI into a Ca^{2+}-independent ATP-binding protein (Hosaka and Sudhof, 1998). For these reasons, we decided to study the functional effect of the ablation of the Ca^{2+}-binding site in the SynI protein, by substituting the glutamate residue with a lysine. We generated a mutant SynI insensitive to Ca^{2+}, E373K-SynI, that we reintroduced in primary hippocampal neurons obtained from SynI KO mice. It has been demonstrated that Syns act as linkers among SVs, and between SVs and the actin cytoskeleton, maintaining SVs in the RP under non-stimulation conditions (Cesca et al. 2010). Our first observation was that the ablation of the glutamate residue induced an enhancement in mEPSC frequency, without any changes in excitatory synapse density. We can hypothesize that, under physiological conditions, SynI acts as a Ca^{2+} buffer, regulating the concentration of this ion in the presynaptic compartment. In absence of the glutamate residue, Ca^{2+} could increase sufficiently to induce the occurrence of miniature events. Even if spontaneous release is independent of APs, it has been already suggested that a part of it is Ca^{2+}-dependent (Simkus and Stricker 2002, Neher and Sakaba 2008). In fact, the presence of BAPTA-AM completely abolishes the previously observed mEPSC increase. Since the mIPSC frequency was not affected, it seems that this alteration in presynaptic residual Ca^{2+} concentration in mutant neurons could preferentially affect excitatory synapses (Fig. 13). Despite SynI being expressed in all neurons (Ferreira et al. 1998), the actual amount of SynI could change between excitatory and inhibitory synapses. Strong experimental evidence shows that SynI expression is particularly heterogeneous in different brain areas and neuronal subtypes (Mandell et al. 1992, Forte et al. 2019, Lugara et al. 2019). The different expression of SynI between excitatory and inhibitory synapses could be the key to understanding the complex pattern of functional changes that we have characterized in both excitatory and inhibitory synapses expressing mutant SynI. It is known that SynI, during HFS, gets

phosphorylated and dissociates from the RP permitting SVs to reach the active zone (Cesca et al. 2010). So, we decided to investigate the role of Ca^{2+} in ATP binding during evoked synaptic transmission. In 2014, Orlando and collaborators investigated the role of ATP in the regulation of SynI function. Using MD simulation, they studied a mutant SynI insensitive to ATP (K269Q-SynI) and our E373K-SynI in the presence or absence of ATP/Ca^{2+} as well as in the presence of ATP but in absence of Ca^{2+}. They held in consideration Hosaka and Sudhof's theory, demonstrating that the presence of Ca^{2+} positively regulated ATP-binding to SynI, but was not necessary for ATP binding to occur. Since the SynI tetramer is less capable of cross-linking adjacent SVs in clusters than the dimer (Cheetham et al. 2001), they also demonstrated that, in absence of Ca^{2+}, SynI dimerization and SV clustering were increased; however, although Ca^{2+} favored the formation of tetramers in expense of dimers, SV clustering was not affected. The authors also observed that the removal of the Ca^{2+}-binding site (E373K-SynI) reduced the capability of ATP to bind SynI and stabilized the MFL (Fig. 12) (Orlando et al. 2014). This latter assumption brought to speculate that the loop stabilization, induced by E373K-SynI, could impair SV clustering.

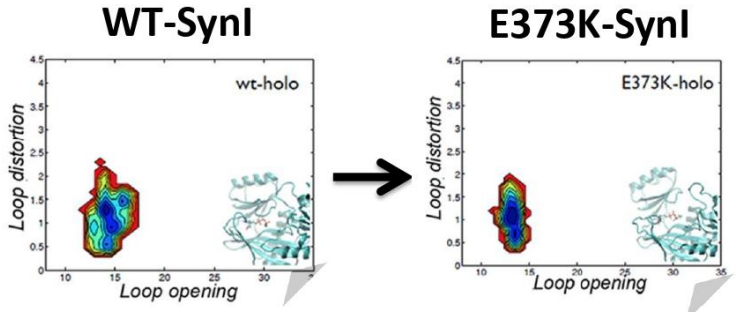


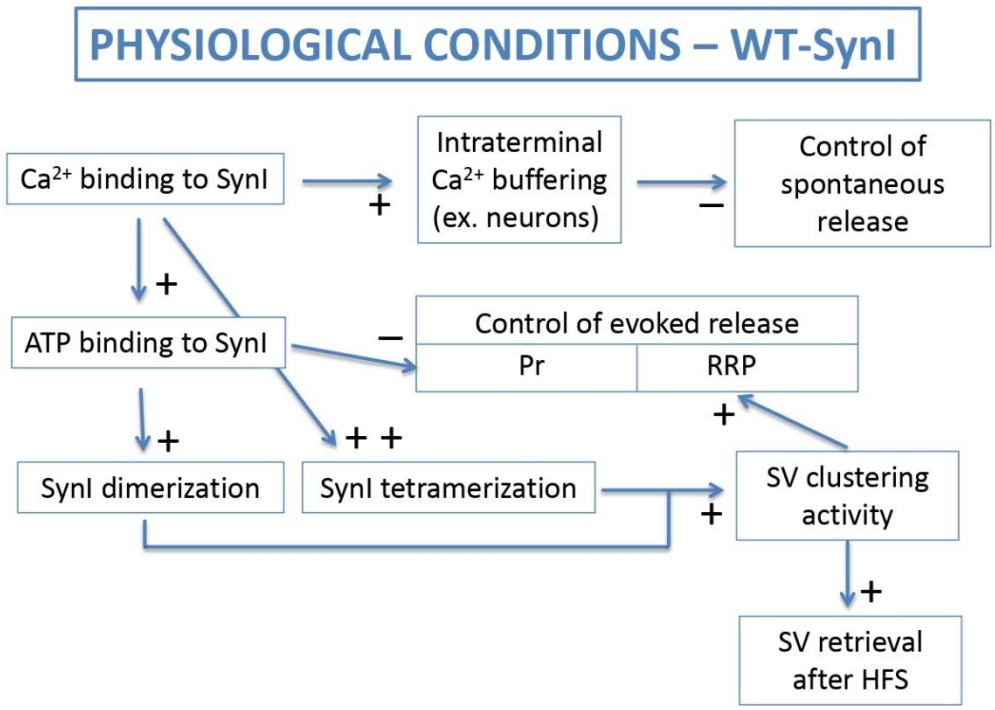
Figure 12. Reduced mobility of Multi-Functional Loop (MFL) in E373K-SynI. Free energy surfaces describing the conformations of the MFL calculated from MD simulations. The reaction coordinates used are the distance between the centers of mass of the MFL residues 336 – 338 and residue 315 (*x*-axis), and the root mean square distance (RMSD) of

the MFL with respect to its conformation in the crystal structure (*y*-axis). WT-SynI with ATP and Ca^{2+} (holo; left), E373K mutant with ATP and Ca^{2+} (holo, right) (Modified from Orlando et al. 2014).

Starting from this point, we tested short term plasticity by stimulating neurons both with short- and long-lasting HFS. Excitatory synapses transduced with E373K-SynI showed a significant reduction of the evoked current amplitude due to a parallel decrease in the RRP_{syn}. On the contrary, inhibitory synapses expressing mutated SynI did not show any difference in terms of amplitude, RRP_{syn} and release probability (Pr) compared to those expressing WT-SynI. We first hypothesized that the absence of Ca^{2+} binding (due to the ablation of the glutamate residue) affects SV clustering, and this mechanism could be at the basis of the RRP_{syn} and amplitude reduction in excitatory synapses. However, the alterations in SV clustering seem not to occur in inhibitory synapses transduced with mutant SynI. Interestingly, both inhibitory and excitatory synapses expressing the E373K-SynI mutation failed to recover after stimulation with long high-frequency trains. Studying SV pool organization and distribution, we discovered that, in E373K-SynI synapses, SV clusters were also profoundly altered. In particular, mutated SynI caused a significant reduction of SV density in excitatory synapses under basal conditions, without affecting inhibitory ones. Both excitatory and inhibitory synapses presented a strong reduction of SV density after 150 s of recovery. It seems that the decreased SynI dimerization and tetramerization induced by the absence of Ca^{2+} binding impaired the ability to reconstitute the SV clusters after endocytosis in both excitatory and inhibitory synapses (Fig. 13). This phenomenon could be at the basis of the depletion of total SVs. In this respect, it has been proposed that SynI could act at endocytic sites to recruit endocytosed SVs, favoring the reconstitution of SV pools (Bloom et al. 2003, Evergren et al. 2007). Ca^{2+} binding may be necessary for this process. Despite the

strong impairment in excitatory synaptic transmission caused by the ablation of the Ca^{2+}-binding site, no significant changes were observed in terms of network firing and/or bursting activity recorded through multi electrode array analysis. A recent work demonstrated the capability of Syns to form a distinct liquid phase in the presynaptic compartment. Milovanovic and collaborators demonstrated that SynI condenses into liquid droplets in vitro. As is already known, lipid vesicles and other SV cluster components specifically partition into these condensates, suggesting that SV cluster formation occurs through protein phase separation. The authors found that SynI phase-separates out of solution under physiological conditions, and this phase separation may generate a reservoir for various synaptic proteins [suggested before by (Milovanovic and De Camilli 2017)]. When they portioned fluorescently labeled liposomes into SynI droplets, they observed the formation of clusters. But when they added ATP and CaMKII, SynI droplets dissolved. Under physiological conditions CaMKII is known to bind and phosphorylate SynI, promoting its dissociation from SVs (Benfenati et al. 1992). Their data supports the idea that SV clusters are condensed droplets that are formed and dissolved in response to physiological cues (Milovanovic et al. 2018). Considering the importance of the Ca^{2+}-binding site for SV clustering, we hypothesize that the Ca^{2+} non-binding could impair SynI phase-separation and lead to the SV cluster disintegration. In conclusion, we have shown that Ca^{2+} binding to SynI is fundamental for correct SV organization and reconstitution after strong depression, suggesting a possible central and not yet demonstrated role of Syns in SV recycling. Excitatory neurons seem to be much more sensitive to the ablation of the glutamate residue; this suggests that excitatory synapses are more susceptible to Ca^{2+} dependent activity of SynI, or that SynI plays a much more pronounced role in its homeostasis. These data represent a starting point

for the understanding of the Ca^{2+}-dependent role of SynI in endocytosis and recycling.



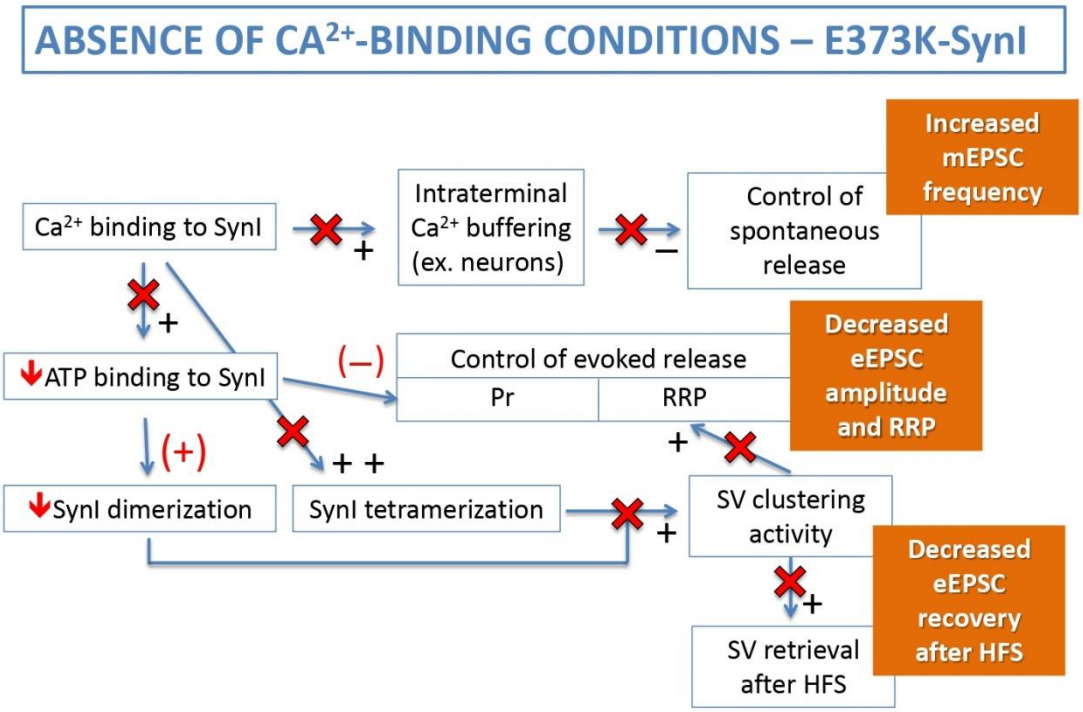


Figure 13. Schematic summary of SynI function in presence and absence of Ca^{2+}-binding site. Under physiological conditions, WT-SynI acts as a Ca^{2+} buffer, controlling the concentration of this ion at the presynaptic terminal. In presence of Ca^{2+}, ATP-binding to SynI is favored; SynI dimerization, tetramerization and SV clustering increase, positively regulating SV release and retrieval after HFS (top). In the presence of E373K-SynI, Ca^{2+} concentration increases, enhancing mEPSC frequency. ATP-binding to SynI is reduced; and consequently dimerization, tetramerization and SV clustering are impaired. eEPSC amplitude and RRP_{syn} decrease and synapses fail to recover after sustained HFS (bottom).

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8. REFERENCES

Akbergenova, Y. and M. Bykhovskaia (2007). "Synapsin maintains the reserve vesicle pool and spatial segregation of the recycling pool in Drosophila presynaptic boutons." Brain Res **1178**: 52-64.

Augustine, G. J. (2001). "How does calcium trigger neurotransmitter release?" Curr Opin Neurobiol **11**(3): 320-326.

Augustine, G. J., M. P. Charlton and S. J. Smith (1985). "Calcium entry and transmitter release at voltage-clamped nerve terminals of squid." J Physiol **367**: 163-181.

Avagliano, L., A. M. Marconi, M. Candiani, A. Barbera and G. Bulfamante (2010). "Thrombosis of the umbilical vessels revisited. An observational study of 317 consecutive autopsies at a single institution." Hum Pathol **41**(7): 971-979.

Baldelli, P., A. Fassio, F. Valtorta and F. Benfenati (2007). "Lack of synapsin I reduces the readily releasable pool of synaptic vesicles at central inhibitory synapses." J Neurosci **27**(49): 13520-13531.

Bear, M. F., B. W. Connors and M. A. Paradiso (2016). Neuroscience : exploring the brain.

Bekkers, J. M. and C. F. Stevens (1991). "Excitatory and inhibitory autaptic currents in isolated hippocampal neurons maintained in cell culture." Proc Natl Acad Sci U S A **88**(17): 7834-7838.

Benfenati, F., F. Valtorta, J. L. Rubenstein, F. S. Gorelick, P. Greengard and A. J. Czernik (1992). "Synaptic vesicle-associated Ca2+/calmodulin-dependent protein kinase II is a binding protein for synapsin I." Nature **359**(6394): 417-420.

Bennett, M. V. L. and R. S. Zukin (2004). "Electrical Coupling and Neuronal Synchronization in the Mammalian Brain." Neuron **41**(4): 495-511.

Bloom, O., E. Evergren, N. Tomilin, O. Kjaerulff, P. Low, L. Brodin, V. A. Pieribone, P. Greengard and O. Shupliakov (2003). "Colocalization of synapsin and actin during synaptic vesicle recycling." J Cell Biol **161**(4): 737-747.

Brenes, O., D. H. Vandael, E. Carbone, P. G. Montarolo and M. Ghirardi (2015). "Knock-down of synapsin alters cell excitability and action potential waveform by potentiating BK and voltage-gated Ca(2+) currents in Helix serotonergic neurons." Neuroscience **311**: 430-443.

Brew, H. M. and I. D. Forsythe (1995). "Two voltage-dependent K+ conductances with complementary functions in postsynaptic integration at a central auditory synapse." J Neurosci **15**(12): 8011-8022.

Candiani, S., L. Moronti, R. Pennati, F. De Bernardi, F. Benfenati and M. Pestarino (2010). "The synapsin gene family in basal chordates: evolutionary perspectives in metazoans." BMC Evol Biol **10**: 32.

Cesca, F., P. Baldelli, F. Valtorta and F. Benfenati (2010). "The synapsins: key actors of synapse function and plasticity." Prog Neurobiol **91**(4): 313-348.

Cheetham, J. J., S. Hilfiker, F. Benfenati, T. Weber, P. Greengard and A. J. Czernik (2001). "Identification of synapsin I peptides that insert into lipid membranes." Biochem J **354**(Pt 1): 57-66.

Chi, P., P. Greengard and T. A. Ryan (2003). "Synaptic vesicle mobilization is regulated by distinct synapsin I phosphorylation pathways at different frequencies." Neuron **38**(1): 69-78.

Chiappalone, M., S. Casagrande, M. Tedesco, F. Valtorta, P. Baldelli, S. Martinoia and F. Benfenati (2009). "Opposite changes in glutamatergic and GABAergic transmission underlie the diffuse hyperexcitability of synapsin I-deficient cortical networks." Cereb Cortex **19**(6): 1422-1439.

Chiappalone, M., A. Vato, L. Berdondini, M. Koudelka-Hep and S. Martinoia (2007). "Network dynamics and synchronous activity in cultured cortical neurons." Int J Neural Syst **17**(2): 87-103.

Cibelli, G., M. Ghirardi, F. Onofri, A. Casadio, F. Benfenati, P. G. Montarolo and F. Vitiello (1996). "Synapsin-like molecules in Aplysia punctata and Helix pomatia: identification and distribution in the nervous system and during the formation of synaptic contacts in vitro." Eur J Neurosci **8**(12): 2530-2543.

Citri, A. and R. C. Malenka (2008). "Synaptic plasticity: multiple forms, functions, and mechanisms." Neuropsychopharmacology **33**(1): 18-41.

Coleman, W. L. and M. Bykhovskaia (2009). "Synapsin I accelerates the kinetics of neurotransmitter release in mouse motor terminals." Synapse **63**(6): 531-533.

Connors, B. W. and M. A. Long (2004). "Electrical synapses in the mammalian brain." Annu Rev Neurosci **27**: 393-418.

Corradi, A., M. Fadda, A. Piton, L. Patry, A. Marte, P. Rossi, M. Cadieux-Dion, J. Gauthier, L. Lapointe, L. Mottron, F. Valtorta, G. A. Rouleau, A. Fassio, F. Benfenati and P. Cossette (2014). "SYN2 is an autism predisposing gene: loss-of-function mutations alter synaptic vesicle cycling and axon outgrowth." Hum Mol Genet **23**(1): 90-103.

De Palma, M. and L. Naldini (2002). "Transduction of a gene expression cassette using advanced generation lentiviral vectors." Methods Enzymol **346**: 514-529.

Etholm, L. and P. Heggelund (2009). "Seizure elements and seizure element transitions during tonic-clonic seizure activity in the synapsin I/II double knockout mouse: a neuroethological description." Epilepsy Behav **14**(4): 582-590.

Evergren, E., F. Benfenati and O. Shupliakov (2007). "The synapsin cycle: a view from the synaptic endocytic zone." J Neurosci Res **85**(12): 2648-2656.

Farisello, P., D. Boido, T. Nieus, L. Medrihan, F. Cesca, F. Valtorta, P. Baldelli and F. Benfenati (2013). "Synaptic and extrasynaptic origin of the excitation/inhibition imbalance in the hippocampus of synapsin I/II/III knockout mice." Cereb Cortex **23**(3): 581-593.

Fassio, A., L. Patry, S. Congia, F. Onofri, A. Piton, J. Gauthier, D. Pozzi, M. Messa, E. Defranchi, M. Fadda, A. Corradi, P. Baldelli, L. Lapointe, J. St-Onge, C. Meloche, L. Mottron, F. Valtorta, D. Khoa Nguyen, G. A. Rouleau, F. Benfenati and P. Cossette (2011). "SYN1 loss-of-function mutations in autism and partial epilepsy cause impaired synaptic function." Hum Mol Genet **20**(12): 2297-2307.

Feliciano, P., H. Matos, R. Andrade and M. Bykhovskaia (2017). "Synapsin II Regulation of GABAergic Synaptic Transmission Is Dependent on Interneuron Subtype." J Neurosci **37**(7): 1757-1771.

Feng, J., P. Chi, T. A. Blanpied, Y. Xu, A. M. Magarinos, A. Ferreira, R. H. Takahashi, H. T. Kao, B. S. McEwen, T. A. Ryan, G. J. Augustine and P. Greengard (2002). "Regulation of neurotransmitter release by synapsin III." J Neurosci **22**(11): 4372-4380.

Ferreira, A., L. S. Chin, L. Li, L. M. Lanier, K. S. Kosik and P. Greengard (1998). "Distinct roles of synapsin I and synapsin II during neuronal development." Mol Med **4**(1): 22-28.

Ferreira, A., H. Q. Han, P. Greengard and K. S. Kosik (1995). "Suppression of synapsin II inhibits the formation and maintenance of synapses in hippocampal culture." Proc Natl Acad Sci U S A **92**(20): 9225-9229.

Ferreira, A., H. T. Kao, J. Feng, M. Rapoport and P. Greengard (2000). "Synapsin III: developmental expression, subcellular localization, and role in axon formation." J Neurosci **20**(10): 3736-3744.

Ferreira, A. and M. Rapoport (2002). "The synapsins: beyond the regulation of neurotransmitter release." Cell Mol Life Sci **59**(4): 589-595.

Fioravante, D. and W. G. Regehr (2011). "Short-term forms of presynaptic plasticity." Curr Opin Neurobiol **21**(2): 269-274.

Fisher, R. S., W. van Emde Boas, W. Blume, C. Elger, P. Genton, P. Lee and J. Engel, Jr. (2005). "Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE)." Epilepsia **46**(4): 470-472.

Fisher, S. A., T. M. Fischer and T. J. Carew (1997). "Multiple overlapping processes underlying short-term synaptic enhancement." Trends Neurosci **20**(4): 170-177.

Forte, N., F. Binda, A. Contestabile, F. Benfenati and P. Baldelli (2019). "Synapsin I Synchronizes GABA Release in Distinct Interneuron Subpopulations." Cereb Cortex.

Garcia, C. C., H. J. Blair, M. Seager, A. Coulthard, S. Tennant, M. Buddles, A. Curtis and J. A. Goodship (2004). "Identification of a mutation in synapsin I, a synaptic vesicle protein, in a family with epilepsy." J Med Genet **41**(3): 183-186.

Giovedi, S., P. Vaccaro, F. Valtorta, F. Darchen, P. Greengard, G. Cesareni and F. Benfenati (2004). "Synapsin is a novel Rab3 effector protein on small synaptic vesicles. I. Identification and characterization of the synapsin I-Rab3 interactions in vitro and in intact nerve terminals." J Biol Chem **279**(42): 43760-43768.

Gitler, D., Q. Cheng, P. Greengard and G. J. Augustine (2008). "Synapsin IIa controls the reserve pool of glutamatergic synaptic vesicles." J Neurosci **28**(43): 10835-10843.

Gitler, D., Y. Xu, H. T. Kao, D. Lin, S. Lim, J. Feng, P. Greengard and G. J. Augustine (2004). "Molecular determinants of synapsin targeting to presynaptic terminals." J Neurosci **24**(14): 3711-3720.

Goda, Y. and T. C. Sudhof (1997). "Calcium regulation of neurotransmitter release: reliably unreliable?" Curr Opin Cell Biol **9**(4): 513-518.

Goelz, S. E., E. J. Nestler, B. Chehrazi and P. Greengard (1981). "Distribution of protein I in mammalian brain as determined by a detergent-based radioimmunoassay." Proc Natl Acad Sci U S A **78**(4): 2130-2134.

Hagler, D. J., Jr. and Y. Goda (2001). "Properties of synchronous and asynchronous release during pulse train depression in cultured hippocampal neurons." J Neurophysiol **85**(6): 2324-2334.

Han, H. Q., R. A. Nichols, M. R. Rubin, M. Bahler and P. Greengard (1991). "Induction of formation of presynaptic terminals in neuroblastoma cells by synapsin IIb." Nature **349**(6311): 697-700.

Hilfiker, S., F. Benfenati, F. Doussau, A. C. Nairn, A. J. Czernik, G. J. Augustine and P. Greengard (2005). "Structural domains involved in the regulation of transmitter release by synapsins." J Neurosci **25**(10): 2658-2669.

Hilfiker, S., F. E. Schweizer, H. T. Kao, A. J. Czernik, P. Greengard and G. J. Augustine (1998). "Two sites of action for synapsin domain E in regulating neurotransmitter release." Nat Neurosci **1**(1): 29-35.

Hosaka, M. and T. C. Sudhof (1998). "Synapsin III, a novel synapsin with an unusual regulation by Ca2+." J Biol Chem **273**(22): 13371-13374.

Hosaka, M. and T. C. Sudhof (1998). "Synapsins I and II are ATP-binding proteins with differential Ca2+ regulation." J Biol Chem **273**(3): 1425-1429.

Hosaka, M. and T. C. Sudhof (1999). "Homo- and heterodimerization of synapsins." J Biol Chem **274**(24): 16747-16753.

Humeau, Y., F. Doussau, M. R. Popoff, F. Benfenati and B. Poulain (2007). "Fast changes in the functional status of release sites during short-term plasticity: involvement of a frequency-dependent bypass of Rac at Aplysia synapses." J Physiol **583**(Pt 3): 983-1004.

Humeau, Y., F. Doussau, F. Vitiello, P. Greengard, F. Benfenati and B. Poulain (2001). "Synapsin controls both reserve and releasable synaptic vesicle pools during neuronal activity and short-term plasticity in Aplysia." J Neurosci **21**(12): 4195-4206.

Jin, Y. and C. C. Garner (2008). "Molecular mechanisms of presynaptic differentiation." Annu Rev Cell Dev Biol **24**: 237-262.

Jovanovic, J. N., F. Benfenati, Y. L. Siow, T. S. Sihra, J. S. Sanghera, S. L. Pelech, P. Greengard and A. J. Czernik (1996). "Neurotrophins stimulate phosphorylation of synapsin I by MAP kinase and regulate synapsin I-actin interactions." Proc Natl Acad Sci U S A **93**(8): 3679-3683.

Kao, H. T., B. Porton, A. J. Czernik, J. Feng, G. Yiu, M. Haring, F. Benfenati and P. Greengard (1998). "A third member of the synapsin gene family." Proc Natl Acad Sci U S A **95**(8): 4667-4672.

Kao, H. T., B. Porton, S. Hilfiker, G. Stefani, V. A. Pieribone, R. DeSalle and P. Greengard (1999). "Molecular evolution of the synapsin gene family." J Exp Zool **285**(4): 360-377.

Katz, B. and R. Miledi (1968). "The role of calcium in neuromuscular facilitation." J Physiol **195**(2): 481-492.

Klagges, B. R., G. Heimbeck, T. A. Godenschwege, A. Hofbauer, G. O. Pflugfelder, R. Reifegerste, D. Reisch, M. Schaupp, S. Buchner and E. Buchner (1996). "Invertebrate synapsins: a single gene codes for several isoforms in Drosophila." J Neurosci **16**(10): 3154-3165.

Lakhan, R., J. Kalita, U. K. Misra, R. Kumari and B. Mittal (2010). "Association of intronic polymorphism rs3773364 A>G in synapsin-2 gene with idiopathic epilepsy." Synapse **64**(5): 403-408.

Leung, P. S., G. P. Brennan, D. W. Halton, C. Shaw, A. G. Maule and G. B. Irvine (1994). "Immunocytochemical localization of neuropeptide F-immunoreactivity in the circumoesophageal ganglia of the gastropod mollusc, Helix aspersa using electron microscopy." Tissue Cell **26**(1): 115-122.

Levine, D. N. (2007). "Sherrington's "The Integrative action of the nervous system": a centennial appraisal." J Neurol Sci **253**(1-2): 1-6.

Li, X., T. W. Rosahl, T. C. Sudhof and U. Francke (1995). "Mapping of synapsin II (SYN2) genes to human chromosome 3p and mouse chromosome 6 band F." Cytogenet Cell Genet **71**(3): 301-305.

Lignani, G., A. Raimondi, E. Ferrea, A. Rocchi, F. Paonessa, F. Cesca, M. Orlando, T. Tkatch, F. Valtorta, P. Cossette, P. Baldelli and F. Benfenati (2013). "Epileptogenic Q555X SYN1 mutant triggers imbalances in release dynamics and short-term plasticity." Hum Mol Genet **22**(11): 2186-2199.

Llinas, R., J. A. Gruner, M. Sugimori, T. L. McGuinness and P. Greengard (1991). "Regulation by synapsin I and Ca(2+)-calmodulin-dependent protein kinase II of the transmitter release in squid giant synapse." J Physiol **436**: 257-282.

Llinas, R., T. L. McGuinness, C. S. Leonard, M. Sugimori and P. Greengard (1985). "Intraterminal injection of synapsin I or calcium/calmodulin-dependent protein kinase II alters neurotransmitter release at the squid giant synapse." Proc Natl Acad Sci U S A **82**(9): 3035-3039.

Lugara, E., A. De Fusco, G. Lignani, F. Benfenati and Y. Humeau (2019). "Synapsin I Controls Synaptic Maturation of Long-Range Projections in the Lateral Amygdala in a Targeted Selective Fashion." Front Cell Neurosci **13**: 220.

Mandell, J. W., A. J. Czernik, P. De Camilli, P. Greengard and E. Townes-Anderson (1992). "Differential expression of synapsins I and II among rat retinal synapses." J Neurosci **12**(5): 1736-1749.

McGuinness, T. L., S. T. Brady, J. A. Gruner, M. Sugimori, R. Llinas and P. Greengard (1989). "Phosphorylation-dependent inhibition by synapsin I of organelle movement in squid axoplasm." J Neurosci **9**(12): 4138-4149.

Medrihan, L., F. Cesca, A. Raimondi, G. Lignani, P. Baldelli and F. Benfenati (2013). "Synapsin II desynchronizes neurotransmitter release at inhibitory synapses by interacting with presynaptic calcium channels." Nat Commun **4**: 1512.

Meinrenken, C. J., J. G. Borst and B. Sakmann (2002). "Calcium secretion coupling at calyx of Held governed by nonuniform channel-vesicle topography." J Neurosci **22**(5): 1648-1667.

Milovanovic, D. and P. De Camilli (2017). "Synaptic Vesicle Clusters at Synapses: A Distinct Liquid Phase?" Neuron **93**(5): 995-1002.

Milovanovic, D., Y. Wu, X. Bian and P. De Camilli (2018). "A liquid phase of synapsin and lipid vesicles." Science **361**(6402): 604-607.

Monaldi, I., M. Vassalli, A. Bachi, S. Giovedi, E. Millo, F. Valtorta, R. Raiteri, F. Benfenati and A. Fassio (2010). "The highly conserved synapsin domain E mediates synapsin dimerization and phospholipid vesicle clustering." Biochem J **426**(1): 55-64.

Neher, E. and T. Sakaba (2008). "Multiple roles of calcium ions in the regulation of neurotransmitter release." Neuron **59**(6): 861-872.

Orlando, M., G. Lignani, L. Maragliano, A. Fassio, F. Onofri, P. Baldelli, S. Giovedi and F. Benfenati (2014). "Functional role of ATP binding to synapsin I in synaptic vesicle trafficking and release dynamics." J Neurosci **34**(44): 14752-14768.

Porton, B., A. Ferreira, L. E. DeLisi and H. T. Kao (2004). "A rare polymorphism affects a mitogen-activated protein kinase site in synapsin III: possible relationship to schizophrenia." Biol Psychiatry **55**(2): 118-125.

Porton, B., H. T. Kao and P. Greengard (1999). "Characterization of transcripts from the synapsin III gene locus." J Neurochem **73**(6): 2266-2271.

Porton, B., W. C. Wetsel and H. T. Kao (2011). "Synapsin III: role in neuronal plasticity and disease." Semin Cell Dev Biol **22**(4): 416-424.

Prasad, D. K., U. Shaheen, U. Satyanarayana, T. S. Prabha, A. Jyothy and A. Munshi (2014). "Association of GABRA6 1519 T>C (rs3219151) and Synapsin II (rs37733634) gene polymorphisms with the development of idiopathic generalized epilepsy." Epilepsy Res **108**(8): 1267-1273.

Richmond, J. (2005). "Synaptic function." WormBook: 1-14.

Rivera, C., J. Voipio, J. A. Payne, E. Ruusuvuori, H. Lahtinen, K. Lamsa, U. Pirvola, M. Saarma and K. Kaila (1999). "The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation." Nature **397**(6716): 251-255.

Rizzoli, S. O. and W. J. Betz (2005). "Synaptic vesicle pools." Nat Rev Neurosci **6**(1): 57-69.

Roberts, W. M. (1993). "Spatial calcium buffering in saccular hair cells." Nature **363**(6424): 74-76.

Rosahl, T. W., D. Spillane, M. Missler, J. Herz, D. K. Selig, J. R. Wolff, R. E. Hammer, R. C. Malenka and T. C. Sudhof (1995). "Essential functions of synapsins I and II in synaptic vesicle regulation." Nature **375**(6531): 488-493.

Rosenmund, C. and C. F. Stevens (1996). "Definition of the readily releasable pool of vesicles at hippocampal synapses." Neuron **16**(6): 1197-1207.

Sabatini, B. L. and W. G. Regehr (1996). "Timing of neurotransmission at fast synapses in the mammalian brain." Nature **384**(6605): 170-172.

Sakaba, T. and E. Neher (2001). "Calmodulin mediates rapid recruitment of fast-releasing synaptic vesicles at a calyx-type synapse." Neuron **32**(6): 1119-1131.

Schiavo, G., F. Benfenati, B. Poulain, O. Rossetto, P. Polverino de Laureto, B. R. DasGupta and C. Montecucco (1992). "Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin." Nature **359**(6398): 832-835.

Schneggenburger, R., A. C. Meyer and E. Neher (1999). "Released fraction and total size of a pool of immediately available transmitter quanta at a calyx synapse." Neuron **23**(2): 399-409.

Schneggenburger, R. and E. Neher (2005). "Presynaptic calcium and control of vesicle fusion." Curr Opin Neurobiol **15**(3): 266-274.

Schoch, S. and E. D. Gundelfinger (2006). "Molecular organization of the presynaptic active zone." Cell Tissue Res **326**(2): 379-391.

Schweizer, F. E. and T. A. Ryan (2006). "The synaptic vesicle: cycle of exocytosis and endocytosis." Curr Opin Neurobiol **16**(3): 298-304.

Simkus, C. R. and C. Stricker (2002). "The contribution of intracellular calcium stores to mEPSCs recorded in layer II neurones of rat barrel cortex." J Physiol **545**(2): 521-535.

Song, S. H. and G. J. Augustine (2015). "Synapsin Isoforms and Synaptic Vesicle Trafficking." Mol Cells **38**(11): 936-940.

Stevens, C. F. and J. H. Williams (2007). "Discharge of the readily releasable pool with action potentials at hippocampal synapses." J Neurophysiol **98**(6): 3221-3229.

Sudhof, T. C. (2004). "The synaptic vesicle cycle." Annu Rev Neurosci **27**: 509-547.

Sudhof, T. C., A. J. Czernik, H. T. Kao, K. Takei, P. A. Johnston, A. Horiuchi, S. D. Kanazir, M. A. Wagner, M. S. Perin, P. De Camilli and et al. (1989). "Synapsins:

mosaics of shared and individual domains in a family of synaptic vesicle phosphoproteins." Science **245**(4925): 1474-1480.

Tang, L. T., T. J. Craig and J. M. Henley (2015). "SUMOylation of synapsin Ia maintains synaptic vesicle availability and is reduced in an autism mutation." Nat Commun **6**: 7728.

Vajda, I., J. van Pelt, P. Wolters, M. Chiappalone, S. Martinoia, E. van Someren and A. van Ooyen (2008). "Low-frequency stimulation induces stable transitions in stereotypical activity in cortical networks." Biophys J **94**(12): 5028-5039.

Valente, P., P. Farisello, F. Valtorta, P. Baldelli and F. Benfenati (2017). "Impaired GABAB-mediated presynaptic inhibition increases excitatory strength and alters short-term plasticity in synapsin knockout mice." Oncotarget **8**(52): 90061-90076.

van Pelt, J., P. S. Wolters, M. A. Corner, W. L. Rutten and G. J. Ramakers (2004). "Long-term characterization of firing dynamics of spontaneous bursts in cultured neural networks." IEEE Trans Biomed Eng **51**(11): 2051-2062.

Waites, C. L. and C. C. Garner (2011). "Presynaptic function in health and disease." Trends Neurosci **34**(6): 326-337.

Wheeler, D. B., W. A. Sather, A. Randall and R. W. Tsien (1994). "Distinctive properties of a neuronal calcium channel and its contribution to excitatory synaptic transmission in the central nervous system." Adv Second Messenger Phosphoprotein Res **29**: 155-171.

Wu, L. G., R. E. Westenbroek, J. G. Borst, W. A. Catterall and B. Sakmann (1999). "Calcium channel types with distinct presynaptic localization couple differentially to transmitter release in single calyx-type synapses." J Neurosci **19**(2): 726-736.

Yang-Feng, T. L., L. J. DeGennaro and U. Francke (1986). "Genes for synapsin I, a neuronal phosphoprotein, map to conserved regions of human and murine X chromosomes." Proc Natl Acad Sci U S A **83**(22): 8679-8683.

Yoshihara, M., B. Adolfsen and J. T. Littleton (2003). "Is synaptotagmin the calcium sensor?" Curr Opin Neurobiol **13**(3): 315-323.

Zucker, R. S. and W. G. Regehr (2002). "Short-term synaptic plasticity." Annu Rev Physiol **64**: 355-405.

9. APPENDIX

Paper published as coauthor by Matteo Moschetta during the PhD course:

The epilepsy-associated protein TBC1D24 is required for normal development, survival and vesicle trafficking in mammalian neurons.

Finelli MJ, Aprile D, Castroflorio E, Jeans A, **Moschetta M**, Chessum L, Degiacomi MT, Grasegger J, Lupien-Meilleur A, Bassett A, Rossignol E, Campeau PM, Bowl MR, Benfenati F, Fassio A, Oliver PL.

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