Synaptic localization of SK channels

By

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CERTIFICATE OF APPROVAL

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"My heart is afraid that it will have to suffer,"

the boy told the alchemist one night as they looked up at the moonless sky.

"Tell your heart that the fear of suffering is worse than the suffering itself.

And that no heart has ever suffered when it goes in search of its dreams."

Paulo Coelho, "The Alchemist"

Chapter I

Introduction

Information flow in the CNS is mediated by chemical synapses that translate electrical signals through neurotransmitter release at presynaptic terminals, and convert back into electrical signals generated by postsynaptic receptors in the postsynaptic neuron. These synaptic signals are transmitted and integrated to the cell body and axon initial segment, where action potentials are generated when the threshold is reached. In addition, they can alter the efficiency of synaptic transmission, 'synaptic plasticity', that is the cellular basis of information processing and storage.

Shaffer collateral (SC) excitatory synapses in the *Stratum radiatum* between presynaptic CA3 terminals onto postsynaptic CA1 dendritic spines have been extensively studied as a model system for understanding basal synaptic transmission and plasticity. These synapses are glutamatergic, and several different types of ionotropic and metabotropic glutamate receptors play key roles involved in most of excitatory synaptic transmission. In addition, several types of Ca^{2+}- and voltage-gated ion channels are expressed in dendritic spines and modulate synaptic responses. Here I will discuss mechanisms of excitatory synaptic transmission and plasticity at SC synapses.

Synaptic transmission

Overview

Excitatory synaptic transmission is primarily mediated by the controlled release of glutamate from synaptic vesicles, housed in the specialized axon terminal, and the activation of postsynaptic glutamate receptors, housed in the specialized structures of dendritic spines. When an action potential is delivered to the presynaptic axonal terminal, depolarization opens voltage-gated Ca^{2+} channels (VGCCs), which generates a large and brief presynaptic Ca^{2+} influx and induces the fusion of synaptic vesicles docked and primed at the active zone of the presynaptic plasma membrane. The release of glutamate into the synaptic cleft, approximately 20 nm width (Zuber et al., 2005; Lucic et al., 2005), is induced with high efficiency and precision, then vesicles are recycled via endocytosis and refilled in order to replenish the releasable vesicle pool (Littleton, 2006; Ryan, 2006). Released glutamate rapidly reaches the postsynaptic membrane within less than a millisecond (Raghavachari and Lisman, 2004) and binds to ionotropic glutamate receptors, in most cases alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors (AMPARs) and N-methyl-d-aspartate receptors (NMDARs). The AMPARs open rapidly and generate the fast component of excitatory postsynaptic currents (EPSCs), allowing influx of Na^{+} ion with 20-80% rise time in 50-100 μs, with about ~10 pA amplitude of miniature EPSCs (Magee and Cook, 2000; Raghavachari and Lisman, 2004). AMPARs deactivate relatively rapidly, with deactivation kinetics ~1 ms, and deactivation rates are influenced by auxiliary subunits such at TARPs (transmembrane AMPAR-regulatory proteins) and cornichon proteins (Tomita et al., 2005; Milestein et al., 2007; Schwenk et

al., 2009; Kato et al., 2010). NMDARs do not become permeable immediately after glutamate is bound, but require the AMPARs-mediated depolarization to free extracellular Mg^{2+} block, thus contributing to a later component of the EPSCs.

Glutamate receptors act as the primary postsynaptic receptors at the majority of excitatory synapses in the brain (Dingledine et al., 1999). At SC synapses, presynaptic release of glutamate activates different types of ionotropic receptors and G-protein coupled metabotropic glutamate receptors (mGluRs) in the dendritic spines. Ionotropic receptors are grouped into three classes on the basis of their electrophysiological properties, and amino acid sequence homology: AMPARs, kainate receptors and NMDARs (Hollmann, 1994).

AMPA receptors

AMPARs are ionotropic and are encoded by four different genes, GluA1-4, and are tetrameric assemblies, formed from diheteromers with GluA1/2 and GluA2/3 in hippocampal CA1 pyramidal neurons (Lu et al., 2009). GluA4 expression is restricted to the first postnatal week (Zhu et al., 2000). One key feature of AMPARs is RNA editing of GluA2 subunit mRNAs, which results in the exchange of a glutamine codon for an arginine codon (Q/R editing) in the pore region (Geiger et al., 1995). Editing efficiency is nearly 100%, and the Q/R edited GluA2 subunit has a profound impact on the biophysical properties such as Ca^{2+}

permeability and the magnitude of conductance (Swanson et al., 1997). Bulkier than glutamine, and positively charged arginine residue in the pore limits Ca^{2+} entry, and significantly reduces single channel conductance with a linear current-voltage relationship. In addition, Q/R editing of GluA2 regulates trafficking from the endoplasmic reticulum (ER) to synaptic sites (Greger et al., 2002; Greger et al., 2003). The edited arginine residue of GluA2 serves as a retention signal and influences assembly. Owing to this property, Q/R edited GluA2 is retained in ER as dimers, which provides an available pool for assembly of tetrameric AMPARs. There are likely retention factors, chaperons, in the ER that interact with Q/R edited GluA2, and replace one subunit with GluA1 or GluA3 during tetrameric assembly for the ER exit (Greger et al., 2002; Shepherd and Huganir, 2007). In contrast, GluA2 lacking AMPARs (GluA1 homomeric or GluA1/3 heteromeric channels) are Ca^{2+} permeable and exhibit inwardly rectifying current-voltage relation due to the voltage dependent block by intracellular polyamines at positive membrane potentials (Geiger et al., 1995; Verdoorn et al., 1991; Donevan et al., 1995). Since the majority of AMPARs in CA1 spines contain at least one GluA2 subunit (Lu et al., 2009), AMPARs are permeable to monovalent cations (Na^+ and K^+), but impermeable to Ca^{2+} ions, with reversal potentials close to 0 mV. At resting membrane potential, activation of AMPARs allows large Na^+ influx and small K^+ efflux, and the net current change mediates most of the fast depolarizing current of EPSC in CA1 pyramidal neurons.

NMDA receptors

NMDARs are heterotetramers composed of dimers of two identical GluN1 and two GluN2 subunits that can be GluN2A, GluN2B, GluN2C, GluN2D, forming either diheteromeric or triheteromeric receptors (Rauner and Kohr, 2010; Tovar and Westbrook, 1999). Occasionally GluN3 subunits (GluN3A, B) replace GluN2 (Kohr, 2006; Ulbrich et al., 2008).

GluN2 subtypes endow distinct gating and channel properties that influence kinetics and amplitude of NMDAR mediated EPSCs. GluN2A or 2B containing NMDARs exhibit higher single channel conductance, Ca^{2+} permeability, and Mg^{2+} sensitivity than GluN2C or GluN2D containing NMDARs. Incorporation of GluN3 to GluN1/GluN2A reduces the conductance and Ca^{2+} permeability of the channels (Das et al., 1998;Perez-Otano et al., 2001). In addition, GluN2 subtypes endow distinct deactivation kinetics, being fastest for GluN2A ($\tau \sim 50$ ms) compared to 2C and 2B ($\tau \sim 300$ ms) or 2D ($\tau \sim 1.7$ s) (Cull-Candy et al., 2001). Thus, the biophysical properties of NMDARs are determined by the contribution of GluN2 or GluN3 subunits.

NMDARs activate more slowly than AMPARs and the NMDAR mediated component of EPSCs decay within hundreds milliseconds (Forsythe et al., 1988; Lester et al., 1990; Luscher et al., 2012). Due to this slower kinetics of NMDARs than AMPARs, they mediate a slower phase of the EPSC. For that reason, the overall contribution of NMDARs to EPSCs is much larger than that of AMPARs

with a relative total charge transfer of NMDAR/AMPAR of ~4 following synaptic stimulation (Gomperts et al., 1998).

The molecular composition of NMDARs is determined by distinct expression patterns during development and the distribution of different GluN2 subunits. While GluN1 is expressed throughout the brain, different GluN2 subunits have more restricted distribution (Monyer et al., 1994). During development, some GluN2B-containing NMDARs are replaced by GluN2A subunits and the predominant assemblies in adult brain are triheteromeric GluN1/GluN2A/GluN2B receptors (Monyer et al., 1994; Quinlan et al., 1999; Gray et al., 2011; Rauner and Kohr, 2010).

NMDA receptors are 'coincidence detectors'

NMDARs are permeable to Ca^{2+} ions and have complex current-voltage relationships due to the voltage dependence that results from external Mg^{2+} blockade at the outer pore region of the channels (Mayer et al., 1984; Nowak et al., 1984). This block is relieved by depolarization thus allowing Ca^{2+} influx through NMDARs into the spine head. NMDARs activate only when glutamate, released from the presynaptic terminal, is bound and the postsynaptic neuron is depolarized. The postsynaptic depolarization can be triggered by synaptic stimulation evoked AMPAR-mediated depolarization or by back-propagating action potentials. The consequence of this coincidence detection of pre- and

postsynaptic activity through NMDARs leads to nonlinear postsynaptic Ca^{2+} responses. Indeed, Ca^{2+} influx through NMDARs promoted by the spine depolarization is an order of magnitude larger than at resting membrane potential (Sabatini et al., 2002), which is crucial for synaptic plasticity. Since it is the Ca^{2+} influx that determines the direction and magnitude of changes in synaptic strength, NMDARs play an essential role of molecular 'coincidence detector' for synaptic plasticity (Bliss and Collingridge, 1993).

Metabotropic glutamate receptors

There are eight subtypes of G-protein coupled mGluRs (mGluR 1-8) and they are categorized within three groups based on sequence homology, biochemical and pharmacological properties (Dingledine et al., 1999; Anwyl, 2006). The mGluR1 and mGluR5 comprise Group 1 mGluRs, which are coupled to Gq, which in turn activates phospholipase C (PLC) (Ferraguti et al., 2008). PLC activation leads to production of diacylglycerol (DAG) and inositol triphosphate (IP_3), which induces Ca^{2+} release from intracellular stores (Pin and Duvoisin, 1995). Group II (mGluR2, mGluR3) and Group III (mGluR4, mGluR6, mGluR7, mGluR8) receptors activate G_i/G_o, which inhibits the activity of adenylyl cyclase (Conn and Pin, 1997; Gerber et al., 2007). mGluRs are expressed both pre- and post-synaptically (Shigemoto et al., 1997) and are involved in synaptic plasticity by influencing transmitter release (Kobayashi et a., 1996) and AMPARs trafficking (Snyder et al., 2001). More details are discussed below.

Postsynaptic density scaffolds proteins

The subspine distributions and mobility of ion channels and receptors are controlled by the arrangement and composition of the postsynaptic density (PSD). The most prominent molecular components of the PSD include scaffold proteins, signaling enzymes, and cytoskeletal elements. A variety of molecular scaffolds and signaling molecules form disk-like proteinaceous structures that determine the dynamic trafficking and stabilization of PSD components. There are three major classes of scaffold proteins in the PSD: the MAGUKs family (membrane-associated guanylate kinases); the SHANK family (SH3 domain and ankyrin repeat domain proteins), also called ProSAPs (proline- rich-synapse-associated proteins); and the Homer family (Scannevin and Huganir, 2000). Among the ten subfamilies of MAGUK proteins, DLG (Drosophila disc large tumor suppressor), MPP (membrane protein palmitoylated), CASK (calcium/calmodulin-dependent serine protein kinase), and CACNB (calcium channel β subunit) are expressed in the central nervous systems (Cho et al., 1992; Kim et al., 1996; Hata et al., 1996; Gosens et al., 2007; Kim et al., 2016). They are highly modular proteins composed of multiple protein-protein interaction domains, containing PDZ (post synaptic density protein), DLG, ZO (zonula occludens-1), SH3 (Src homology 3), and GK (guanylate kinase-like) domains. All DLG subfamily members, SAP97 (DLG1), PSD-93 (DLG2), SAP102 (DLG3), PSD-95 (DLG4), have three type I PDZ domains, whereas CASK and MPP scaffolds contain type II PDZ domains (Oliva et al., 2011).

PSD-95 and SAP97 strongly interact with GluN2A subunit via PDZ domain interactions with the PDZ ligand domain of the receptors (Bassand et al., 1999). This interaction influences the subcellular distribution of GluN2A-containing NMDARs in PSD (Bassand et al., 1999). The SH3 domain typically interacts with proline-rich sequences, and is known to mediate interactions between PSD-95 and SAP97 (Cai et al., 2006). Primordial GK domains had catalytic activity, transferring phosphate from ATP to GMP, however, this has been lost and the GK domains now serve as protein-protein interaction domains lacking of any detectable catalytic activity (Kuhlendahl et al., 1998; Li et al., 2002).
Some subclasses of DLG (SAP97, PSD-93, PSD-95), CASK, and MPP (2, 5, 7) include L27 domain (found in Lin-2 and Lin-7) near the N-terminus, which mediate homo or heterophilic multimerization with other proteins via L27 domains (Feng et al., 2004; Li et al., 2004).

The Shank family contains ankyrin repeats, an SH3 domain, a PDZ domain, a proline-rich domain, and a sterile alpha motif (SAM) domain. Shank proteins homo-multimerize and form sheet like structures that serve as a platform for higher order organization of the PSD (Baron et al., 2006). Shank proteins have multiple binding partners such as SAP97 (Regalado et al., 2006), Homer (Tu et al., 1999), membrane-cytoskeletal protein α-fodrin (Bockers et al., 2001), PKC-binding protein Sharpin (Lim et al., 2001), and Rac1/Cdc42 GEF (Park et al., 2003). Thus, Shank proteins form homomultimers and act as synaptic backbones

for crosslinking PSD receptors, other scaffolding proteins, and signaling molecules.

Homer protein contains the large family of EVH1 (Ena/VASP homology 1) domain and leucine-zipper motifs that have coiled-coil (CC) structures at the C-ternimus. The CC domain is responsible for multimerization (Brakeman et al., 1997; Tadokoro et al., 1999). The EVH1 domain mediates interaction with the cytosolic domain of mGluRs (Tu et al., 1998). Furthermore, Homer proteins bind to IP_3 receptors (Tu et al., 1999), Shank, and Cav2.1 (Okabe et al., 2001).

Thus, the structure of PSD includes a variety of scaffold proteins that is closely coupled to the membrane receptors, ion channels, enzymes and signaling molecules. These scaffold proteins contain multiple protein interaction modules and serve as a synaptic backbone in the PSD. This web-like protein networks provide multiple binding slots for synaptic receptors and cytoplasmic signaling enzymes. Thus, PSD scaffold proteins form the basis of the organization of the PSD, influencing the arrangement, anchoring and clustering of synaptic receptors. The role of PSD scaffold proteins on the localization of glutamate receptors will be discussed in below.

The distribution of glutamate receptors

NMDARs are highly enriched in the PSD where they are clustered within the central portion of the PSD (Racca et al., 2000), although there is a mobile pool that travels between the extra- and postsynaptic membrane domains (Groc et al., 2004; Tovar and Westbrook, 2002). This differential targeting is due to specific interaction of scaffold proteins with different subunits of NMDARs. PSD-95 is highly abundant in the PSD (Cheng et al., 2006) and interacts with GluN2A subunit, and this preferential association influences synaptic distribution and clustering of GluN2A-containing NMDARs as evidenced by co-immunoprecipitation of PSD-95 with GluN2A from the hippocampus and heterologous expression systems (Bassand et al., 1999; Chen et al., 2011). Imaging analysis shows colocalization of PSD-95 and GluN2A in hippocampal neuronal cultures, which is disrupted by PSD-95 knock down (Chen et al., 2011). In contrast, GluN2B-containing NMDARs are highly mobile and are found in both synaptic and extrasynaptic membranes (Groc et al., 2004; Tovar and Westbrook, 2002). SAP102 is evenly distributed between extrasynaptic and synaptic membrane, and associates with GluN2B (Sans et al., 2000; Washbourne et al., 2004; Sanz-Clemente et al., 2010). SAP102 forms a complex with GluN2B in brain homogenates as shown by co-IP, and SAP102 colocalizes with GluN2B throughout the synaptic and extrasynaptic membranes (Sans et al., 2000; Sans et al., 2003; Washbourne et al., 2004)

AMPARs are highly mobile and continuously exchange between the extrasynaptic and synaptic membranes. They freely diffuse in and out the synapse under basal conditions, then GluA1 containing AMPARs are inserted into synapses during synaptic activity (Carroll et al., 1999; Luscher et al., 1999; Heine et al., 2008). This mobility of AMPARs is determined by interaction between their auxiliary subunits such as TARPs and synaptic scaffold proteins such as PSD-95 and SAP97.

Stargazin (TARP γ-2) directly interacts with AMPARs and promotes their transport to the cell surface and synapse (Chen et al., 2000; Tomita et al., 2005). In slice cultures of Stargazin knockout mice, AMPAR mediated EPSCs are absent, and synaptic labeling of AMPARs is reduced (Chen et al., 2000). Coexpression of Stargazin with GluA1 in *Xenopus* oocytes leads to enhanced glutamate-evoked currents and membrane trafficking of GluA1 with slow desensitization and deactivation. In addition, Stargazin expression in hippocampal slice culture increases the amplitude of AMPAR EPSCs and mEPSCs, supporting the crucial role of Stargazin on the mobility and synaptic function of AMPARs (Tomita et al., 2005).

PSD-95 forms complexes with AMPARs, which stabilizes synaptic AMPARs through binding to the Stargazin (Dakoji et al., 2003; Bats et al., 2007). Stargazin bound AMPARs freely diffuse between extrasynaptic and synaptic membranes, and when PSD-95 binds to Stargazin, it acts to anchor AMPARs in the PSD.

When the interaction of PSD-95 with Stargazin is impaired with the expression of a partial peptide of Stargazin lacking the PDZ ligand in hippocampal neurons in culture, AMPAR clustering in synapses is reduced with increased surface diffusion of AMPARs, supporting the idea that AMPAR stabilization requires interactions with Starazin and PSD-95 (Bats et al., 2007).

SAP97 directly binds to the GluA1 subunit and promotes synaptic localization of AMPARs in CaMKII dependent manner (Leonard et al., 1998; Mauceri et al., 2004). SAP97 preferentially co-immunoprecipitates with GluA1 from rat brain membrane fractions but does not associate with other AMPARs subunits (Leonard et al., 1998). Upon the induction of LTP, CaMKII phosphorylates SAP97 that promotes SAP97 binding to GluA1 containing AMPARs. Blocking of CaMKII dependent phosphorylation of SAP97 impairs synaptic localization and clustering of SAP97 (Mauceri et al., 2004). Expression of SAP97 mutant unable to be phosphorylated by CaMKII impairs synaptic labeling of GluA1 in hippocampal cultures (Mauceri et al., 2004). This activity-dependent synaptic distribution of GluA1-containing AMPARs is determined by interaction with the PSD scaffold proteins that is a major regulatory mechanism in controlling synaptic transmission and plasticity.

The mGluRs do not reside in the PSD but are mobile in the extrasynaptic membrane and dendritic shafts where they are stabilized by the association with Homer (Lujan et al., 1997; Roche et al., 1999; Serge et al., 2002). Homer1

proteins co-immunoprecipitate mGluRs and induce dendritic localization and clustering of group I mGluRs. Co-expression of Homer1 with mGluRs reduces lateral mobility of mGluRs and stabilizes clusters of receptors as shown by single-particle tracking and live cell imaging (Serge et al., 2002). In addition, Homer co-immunoprecipitates with IP_3 receptors located on intracellular Ca^{2+} storages, thereby crosslinking mGluRs and internal Ca^{2+} stores (Tu et al., 1998). This signaling complex provides physical proximity between mGluRs and intracellular Ca^{2+} source that may also contribute functional coupling between receptors and intracellular Ca^{2+} signaling cascade.

Thus, the organization of PSD scaffold proteins stabilizes and confines the distribution of glutamate receptors in the spine. Scaffold proteins often multimerize each other to form protein networks which assembles signaling molecules and enzymes. This regulates activity dependent trafficking of receptors and defines synaptic functions and plasticity.

The Ca^{2+} transient within the spine head

The influx of Ca^{2+} during synaptic transmission is crucial to synaptic transmission and plasticity. Synaptic stimulation increases intracellular Ca^{2+} from three sources; influx through NMDARs, influx through voltage-gated Ca^{2+} channels (VGCCs), and Ca^{2+} release from the intracellular Ca^{2+} stores. Synaptic stimulation produces Ca^{2+} transients in dendritic spines measured by Ca^{2+}

imaging, reaching a maximum of ~700 nM from 50 nM at rest, which lasts over ~100 ms (Sabatini et al., 2002; Bloodgood and Sabatini, 2007). This Ca^{2+} transient in the spine head is mostly generated by Ca^{2+} influx through NMDARs; blocking NMDARs reduces the Ca^{2+} transient by ~80% (Sabatini et al., 2002). Another significant contributor to the spine Ca^{2+} transient is through VGCCs. Particularly R-type Ca^{2+} channels make a major contribution to Ca^{2+} influx into the spine head subsequent to back-propagating action potentials (Bloodgood and Sabatini 2007; Parajuli et al., 2012). R-type Ca^{2+} channels are located in extrasynaptic membranes as shown by immuno-EM (Parajuli et al., 2012), and couple to voltage-gated Kv4.2-containing channels. Thus Cav2.3 R-type Ca^{2+} channels in spine heads serve as a distinct Ca^{2+} source within the dendritic spine (Wang et al., 2014), which will be discussed later. L-type Ca^{2+} channels are also expressed in spines and make a relatively small contribution compared to R-type Ca^{2+} channels in response to back-propagating action potentials (Hoogland and Saggu, 2004). Ca^{2+} imaging studies have shown that L-type Ca^{2+} channels blocker treatment significantly reduces Ca^{2+} transient in dendritic spines with brief bursts of back-propagating action potentials (Hoogland and Saggu, 2004). In addition, L-type Ca^{2+} channels associate with β2-adrenergic receptors (β2-ARs), which requires PKA and CaMKII activity (Davare et al., 2001; Hoogland and Saggu, 2004). CICR (calcium-induced calcium release) is an intracellular Ca^{2+} source activated by mGluRs that can lead to IP_3 production and Ca^{2+} release from the smooth endoplasmic reticulum (SER) by IP_3 receptor activation (Finch

and Augustine, 1998; Miyata et al., 2000; Takechi et al., 1998). Thus, the distinct compartmentalization of Ca^{2+} sources allows functional coupling to specific receptors within discrete nanodomains.

K^+ channels in dendritic spines

Dendritic spines of CA1 pyramidal neurons also contain several types of K^+ channels that influence synaptic responses. These K^+ channels are activated by either membrane depolarization or intracellular Ca^{2+} influx. Small-conductance Ca^{2+} activated K^+ channels (SK channels) are located in the PSD of dendritic spines and are closely associated with NMDARs, forming a nanodomain within ~50 nm (Lin et al., 2008; Fakler and Adelman, 2008). Ca^{2+} influx via NMDARs activates SK2-containing channels, which provide an outward repolarizing K^+ conductance, limiting the EPSP and restoring Mg^{2+} unblock to NMDARs, and thus reducing the Ca^{2+} transient in the spine head (Ngo-Anh et al., 2005; Lin et al., 2008; Wang et al., 2014). SK2 channels also coassemble with mGluR5 in hippocampus and are activated by mGluR5 stimulation via PLC and CICR (García-Negredo et al., 2014). In heterologous expression studies, stimulating mGluR5 activated a SK2 mediated K^+ current, and coexpression of mGluR5 and SK2 channels promoted plasma membrane targeting of both proteins. In addition, immuno-EM on hippocampal slices revealed that extrasynaptic SK2 channels colocalize with mGluR5 receptors in dendritic spines of CA1 neurons, suggesting

that mGluR5 dependent IP_3-mediated Ca^{2+} mobilization is coupled to extrasynaptic SK2 channels.

Voltage-gated Kv4.2-containing channels are also localized to the extrasynaptic membrane within the spine head and are activated by postsynaptic depolarization (Hoffman et al., 1997). Kv4 channels activate and inactivate rapidly on membrane depolarization, and underlie A-type K^+ currents (I_A) (Serodio et al., 1994). They are highly enriched in the dendritic spines of CA1 pyramidal neurons (Kim et al., 2007) and electrophysiological studies along with Ca^{2+} imaging revealed that A-type K^+ channels are present in the apical dendrite and dendritic spines (Hoffman et al., 1997). They are activated by EPSP and back-propagating action potentials, and their activation attenuates the amplitude and width of postsynaptic responses and bAPs (Hoffman et al., 1997; Cai et al., 2004; Kim et al., 2005). When activated by an EPSP or bAPs, Kv4.2 channels rapidly inactivate, leading to larger subsequent EPSPs and back-propagating action potentials, which is important for the induction of some forms of LTP (Watanabe et al., 2002). Recent reports showed that Kv4.2 channels couple to R-type Ca^{2+} channels in the spine and reduce synaptically evoked EPSPs (Wang et al., 2014). Ca^{2+} influx via R-type Ca^{2+} channels serves as a distinct Ca^{2+} source for Kv4.2, mediated by their Ca^{2+} binding KChiP (K^+ channel interacting protein) auxiliary subunits, while SK2-containing channels use NMDARs as an independent Ca^{2+} source in spines (Wang et al., 2014). Thus, Kv4.2 and SK2

containing channels serve a synergistic role in shaping synaptic response and integration.

Ca^{2+}-activated Cl^- channels

Recently it has been shown that Ca^{2+}-activated Cl^- channels (CaCCs) are present in hippocampal neurons and play a role in synaptic function for neuronal signaling (Huang et al., 2012). Transmembrane protein 16B (TMEM16B) was shown to encode CaCCs. Like SK channels, they are in close proximity with NMDARs and use them as a Ca^{2+} source for their activation to dampen EPSP and impede temporal summation.

Synaptic Plasticity at Schaffer collateral synapses

Overview

Synaptic plasticity is the activity dependent change in synaptic strength between neurons. Dynamic changes in synaptic efficacy are believed to underlie the cellular basis for learning and memory in the mammalian brain (Lendvai et al., 2000; Whitlock et al., 2006; Matsuo et al., 2008).

There are various forms of plasticity in hippocampus. Short-term plasticity at hippocampal synapses can be presynaptically expressed by altering transmitter release during a train of action potentials, and reflects residual presynaptic Ca^{2+} levels, rate of vesicle recycling, and transmitter release probability. Such changes

last from a few miliseconds to a minute (Regehr, 2012). Long-term change in the strength of synaptic connectivity is generally accepted as a model of the cellular processes underlying learning and memory, as originally postulated by Hebb (1949) and others (Markram et al., 2011). Long-term plasticity is expressed postsynaptically at SC synapses by modulating the density, types, and properties of receptors and signaling molecules, which lasts hours, days, or even weeks (Bliss and Lomo, 1973; Bliss and Gardner-Medwin, 1973; Staubli and Lynch, 1987; Staubli and Lynch, 1990; Abraham, 2003). Traditionally, LTP is induced by repetitive high-frequency presynaptic stimulation or by pairing low-frequency presynaptic stimulation with postsynaptic depolarization. In contrast, prolonged low-frequency stimulation can induce LTD. Inducing this type of plasticity, either LTP or LTD, in hippocampal CA1 pyramidal neurons requires postsynaptic depolarization and intracellular Ca^{2+} accumulation in the postsynaptic spine head. The rise in Ca^{2+} level in the postsynaptic spine activates many intracellular signaling cascades, which ultimately result in altered synaptic strength (Neveu and Zucker, 1996; Cummings et al., 1996; Pereda et al., 1998).

NMDARs contribute a significant fraction of Ca^{2+} influx into the dendritic spine, which is necessary for inducing synaptic plasticity (Huang et al., 1992; Christie et al., 1996; Schiller et al., 1999). Importantly, NMDAR activation requires glutamate binding and depolarization of the postsynaptic neuron, conferring the property of NMDARs as coincidence detectors. Shaffer collateral to CA1 synapses express

the most robust NMDARs-dependent form of plasticity. Here I will focus on the underlying mechanisms for induction and expression of NMDAR-dependent plasticity.

NMDAR dependent LTP

Induction

Long-term synaptic plasticity at SC synapses requires NMDARs activation, and one important feature of NMDARs dependent LTP is input- or synapse-specificity. This means that only synapses that experience the induction stimulus will undergo the plasticity. In this regard, NMDARs have crucial role as molecular coincidence detectors for correlated activity. Induction of NMDARs dependent synaptic plasticity requires temporally correlated activation of both pre- and postsynaptic neurons.

Moreover, the inducing postsynaptic depolarization depends on the frequency and pattern of stimulation. High frequency of tetanic stimulation, composed of several trains of pulses at 50-100 Hz for 1 sec., can induce LTP. High frequency presynaptic stimulations generate a strong temporal summation of EPSP that is sufficient to relieve Mg^{2+} block of NMDARs. Alternatively, 'pairing' protocols can also induce LTP by depolarizing the spine by holding at a depolarized membrane potential while delivering presynaptic stimulation to precisely control depolarization of the postsynaptic neuron (Markram et al., 1997; Magee and

Johnston, 1997). Correlated pre- and post-synaptic activity that induces LTP can also be provided by pairing back-propagating action potentials in the postsynaptic neuron with presynaptic stimulation. This additional depolarization generated by back-propagating action potential facilitates Ca^{2+} influx through NMDARs (Stuart et al., 1997; Waters et al., 2005). The time window and order between pre- and post-synaptic firing is crucial to determine the direction and magnitude of synaptic plasticity. When repeatedly elicited EPSPs through presynaptic stimulations precede back-propagating action potentials, referred to as “pre-post”, such repetitive “pre-post” firing can induce LTP. While postsynaptic spiking arriving before the EPSP, “post-pre”, induces LTD. This associative form of plasticity is called spike timing-dependent plasticity (STDP) (Dan and Poo, 2006; Caporale and Dan, 2008).

Presynaptic or postsynaptic locus of expression

After LTP was discovered at dentate granule neurons (Bliss and Lomo, 1966), the locus of NMDAR dependent LTP expression, pre- vs postsynaptic, was intensely debated (Nicoll, 2003). In principal, the induction of LTP can lead to increased synaptic strength either by enhanced probability of transmitter release or postsynaptic changes in ion channels and signaling molecules.

Some reports suggested that NMDAR dependent LTP at SC synapses is associated with enhanced presynaptic function. Thus, measuring paired-pulse

ratios before and after induction of LTP showed paired pulse facilitation, reflecting short-term changes in release probability. This short-term facilitation was shown to be associated with LTP expression (Schulz et al., 1994; Schulz, 1997). Also, visualizing presynaptic vesicles with FM1-43 dye was used to examine vesicle recycling. FM1-43 can be loaded into the vesicle as they are endocyotosed and then they destain as they undergo exocytosis. The destaining time course was used to measure the rate of transmitter release, and an increased destaining rate was observed after LTP induction (Zakharenko et al., 2001; Zakharenko et al., 2003; Stanton et al., 2005). Further, MK-801 irreversibly blocks NMDARs (Huettner and Bean, 1988) and gradually decreases EPSCs when glutamate binds to NMDARs. The blocking rate of the EPSCs correlated with the probability of presynaptic transmitter release. It was observed that this blocking rate was increased after LTP induction suggesting increased probability of transmitter release (Kullmann et al., 1996). Finally, the relative ratio of AMPAR and NMDAR components of the EPSC was not changed after LTP, but parallel increases of both components were observed, suggesting increased presynaptic transmitter release (Tsien and Malinow, 1990; Asztely et al., 1992; Muller et al., 1992).

However, contradictory results were obtained for each of these. For example, a lack of change in paired pulse facilitation after LTP (McNaughton, 1982; Manabe et al., 1993; Schulz et al., 1995; Asztely et al., 1996), no change in destaining rate of FM1-43 (Zakharenko, 2001), a lack of change in blocking rate of MK-801

(Zakharenko, 2001), and a selective increase in the AMPAR component of EPSC after LTP were reported (Kauer et al., 1988; Muller et al., 1988; Liao et al., 1995). Despite these contradictions, quantal analysis strongly supported a presynaptic mechanism for LTP expression. Quantal analysis showed a decrease in the coefficient of variation, the failure rate, and increase in quantal content supporting a presynaptic expression mechanism (Malinow and Tsien, 1990; Bekkers and Stevens, 1990; Manabe et al., 1993; Kullmann and Nicoll, 1992; Liao et al., 1992; Stevens and Wang, 1994; Stricker et al., 1996).

The controversy was, however, largely resolved by the discovery of silent synapses on CA1 pyramidal neurons, and this provided a framework for understanding the apparent change in quantal analysis via a postsynaptic mechanism. Silent synapses were those that contained only NMDARs and no, or few, AMPARs (Isaac et al., 1995; Liao et al., 1995; Hsia et al., 1998; Liao et al., 1996; Durand et al., 1996). Electrophysiological recording with minimal synaptic stimulation, which did not elicit EPSCs at –60 mV, elicited a slow EPSC when holding at +30 mV. This EPSC disappeared with the application of the NMDAR antagonist, D-APV, indicating these 'silent synapses' contained only NMDARs (Isaac et al., 1995; Liao et al., 1995). After LTP induction, the minimal stimulation evoked a detectable AMPAR component of EPSCs at –60 mV, demonstrating silent synapses acquire AMPARs in the PSD, then become functional; they undergo "unsilencing" upon the induction of LTP (Isaac et al., 1995; Liao et al., 1995). Furthermore, a number of immunocytochemistry and immunogold EM

studies have found that all excitatory synapses contain NMDARs, but the distribution of AMPARs is highly heterogeneous and subpopulations of dendritic spines lack AMPARs (Nusser et al., 1998; Petralia et al., 1999; Takumi et al., 1999), and activity induces the rapid appearance of AMPARs immunoreactivity (Liao et al., 2001; Lu et al., 2001). This suggested the existence of silent synapses that have normal NMDARs components with no AMPAR component. The unsilencing of silent synapse by adding more synaptic AMPARs can explain the decrease in the frequency of synaptic failures and an increase in the index CV^{-2} (the inverse square of the coefficient of variation of EPSC amplitudes) with the postsynaptic expression mechanism. Synapse unsilencing mimics an increase in "n", the number of synapse activated, which appears consistent with increased quantal content and decreased failure rate. The increased quantal contents without change in "p", probability of release, can account for the absence of change in paired pulse facilitation (McNaughton, 1982; Manabe et al., 1993; Schulz et al., 1995; Asztely et al., 1996). Since then, many studies support postsynaptic expression of LTP via activity dependent trafficking of AMPARs into the PSD of dendritic spine (Luscher and Frerking, 2001; Malenka and Nicoll, 1999; Malinow and Malenka, 2002; Nicoll, 2003; Malenka and Bear, 2004). It is now generally accepted that addition of postsynaptic AMPARs is the major mechanism underlying expression of NMDARs dependent LTP at SC syanpses.

Trafficking of AMPARs

AMPARs are highly mobile and rapidly recycle at the synapse, so the net change in AMPARs trafficking into the synapse contributes to the expression of synaptic plasticity (Malinow and Malenka, 2002). Under basal conditions, GluA2/3 heteromers constitutively cycle in and out of synapses involving SNARE protein mediated exocytosis and dynamin dependent endocytosis (Luscher, 1999; Carroll et al., 2001; Kennedy and Ehlers, 2011), preserving the total number of synaptic AMPARs. In contrast, GluA1 containing AMPAR trafficking is activity dependent and the PDZ ligand at the GluA1 C-terminus is critical for trafficking into synapses, which underlies LTP expression (Shi et al., 1999; Hayashi et al., 2000; Piccini and Malinow, 2002). Synaptic delivery of GluA1 can be visualized by transfecting GFP tagged GluA1 in hippocampal neurons or slice cultures. In addition, increased synaptic trafficking of GluA1-GFP can be measured with electrophysiological recording after LTP induction, exhibiting increased inward rectification (Shi et al., 1999), a signature physiological characteristic of GluA1-containing AMPARs. Heteromeric GluA1/A2 receptors are one of the primary AMPARs subtypes expressed in hippocampus, and are inserted into synapses during LTP (Adesnik and Nicoll, 2007). Furthermore, there is activity dependent subtype exchange of GluA2-containing AMPARs to GluA2 lacking AMPARs (GluA1/GluA3 or GluA1/A1) in synapses. GluA2 lacking AMPARs have two benefits to potentiate synapses. They are Ca^{2+} permeable, thus serve as additional Ca^{2+} source, and have higher conductance that can induce larger potentiation (Liu and Zukin, 2007). Upon the induction of LTP, GluA2 lacking

AMPARs are transiently incorporated into synapses and this is required for the maintenance of NMDAR dependent LTP (Plant et al., 2006). Therefore, increased numbers of synaptic AMPARs account for the major component of increased excitatory responses underlying LTP.

Second messenger signaling in LTP

Protein kinases have critical roles on the activity dependent trafficking of GluA1-containing AMPARs. Ca^{2+} influx via NMDARs activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and this is required for LTP (Lisman et al., 2002). CaMKII phosphorylates at S831 in the GluA1 C-terminus that increases AMPARs conductance (Benke et al., 1998). PKA phosphorylates S835 of GluA1 (Roche et al., 1996) and this phosphorylation is necessary for synaptic delivery of GluA1 to the synapse during LTP (Lee et al., 2000; Lee et al., 2003). PKC phosphorylates S818 and S831 of GluA1 C-tail (Boehm et al., 2006; Roche et al., 1996) and facilitates synaptic AMPARs delivery. Phosphorylation of the GluA1 C-terminus is revealed by immunoblot using phosphorylation-site-specific antibodies, and a phosphor-mimic mutation leads to increased rectification of AMPAR-EPSCs (Lee et al., 2000; Boehm et al., 2006). LTP induction elevates kinase activity, which leads to increased exocytosis of AMPARs into the extrasynaptic membrane. This elevates the available pool of AMPARs in the perisynaptic site, and they translocate into the synapse (Yang et al., 2008). The extrasynaptic population of AMPARs is detected by blocking glial glutamate

transporter, EAAT1 (excitatory amino acid transporter). TBOA (threo-beta-Benzyloxyaspartate), an EAAT inhibitor, treatment enhances EPSPs by blocking glutamate uptake, allowing released glutamate to spill over out of the synapse and activate the perisynaptic population of AMPARs which translocate to the synapse during LTP. Thus perisynaptic pool of AMPARs serves as an intermediate checkpoint for LTP expression.

NMDAR dependent LTD

Induction methods

In contrast to LTP, NMDAR dependent LTD can be induced by repeated low-frequency presynaptic stimulations at 0.1-1 Hz for 5-15 min. This leads to modest depolarization of postsynaptic neurons to relieve Mg^{2+} block of NMDARs. Mild and repeated activation of NMDARs allows prolonged and modest Ca^{2+} levels in the postsynaptic neurons, which are optimal for triggering LTD (Selig et al., 1995). Pairing protocols can also induce LTD by holding postsynaptic neurons at modest depolarized potentials, -30 mV, with low-frequency synaptic stimulation (0.1-1 Hz). This membrane depolarization relieves Mg^{2+} block of NMDARs, and activating NMDARs with low-frequency presynaptic stimulation results in prolonged postsynaptic Ca^{2+} level. Another induction method is pairing synaptic stimulation with back-propagating action potentials, one form of STDP. When the back-propagating action potential is repeatedly generated before presynaptic stimulation, “post-pre”, LTD is observed (Collingridge et al., 2010; Luscher and

Malenka, 2012). Brief application of NMDA can also induce a form of LTD known as chemical LTD, which appears to share common mechanisms with low-frequency stimulation induced LTD (Lee et al., 1998).

Expression mechanisms

Many studies show that NMDAR dependent LTD expression is mainly a postsynaptic mechanism in hippocampal CA1 pyramidal neurons. Particularly, a reduction of the number of synaptic AMPARs is a generally accepted mechanism underlying the expression of NMDAR dependent LTD (Beattie et al., 2000; Lissin et al., 1999; Ehlers, 2000; Malinow and Malenka, 2002; Sheng and Lee; 2003). The net balance of endocytosis and exocytosis of AMPARs at synapses determines the total number of synaptic AMPARs, so an increased rate of endocytosis results in LTD whereas increasing the rate of exocytosis leads to synaptic trafficking of AMPARs and LTP (Kessels and Malinow, 2009).

AMPARs are highly mobile and constitutively recycle between synapse and cytoplasm. Synaptic AMPARs are stabilized by the interaction with NSF (N-ethylmaleimide-sensitive fusion protein). NSF binds to GluA2 and involves SNARE-mediated incorporation and stabilization of synaptic AMPARs (Beretta et al., 2005). AMPAR internalization is mediated by the clathrin adaptor protein, AP-2, which binds to overlapping sites on GluA2 where NSF also binds (Lee et al., 2002). Stabilized AMPARs mediated by NSF interaction are disrupted when AP-2

replaces NSF, and this triggers AMPAR endocytosis that results in LTD (Lee et al., 2002; Collingridge et al., 2004). GRIP/ABP (Glutamate receptor-interacting protein/AMPAR binding protein), and PICK1 (protein interacting with C-kinase) are known to interact with GluA2 via the PDZ domain at their C-termini and regulate membrane trafficking and synaptic targeting of AMPARs. GRIP/ABP promotes recycling of internalized AMPARs back to the synapses (Dong et al., 1997; Srivastava et al., 1998). PICK1 competes for binding to the PDZ ligand of GluA2 with GRIP/ABP, which promotes the internalization of GluA2-containing AMPARs. Dissociation of GRIP/ABP from GluA2 is determined by phosphorylation of GluA2 on S880 where it is phosphorylated by PICK1 bound PKC (Kim et al., 2001; Seidenman et al., 2003). Thus, association of PICK1 with GluA2-containing AMPARs facilitates removal of synaptic AMPARs and mediates LTD expression, whereas association of GRIP/ABP stabilizes synaptic AMPARs by limiting PICK1 mediated AMPARs endocytosis (Osten et al., 2000; Shi et al., 2001).

Phosphatase activity is necessary for LTD by reversing the phosphorylation status of GluA1, which removes AMPARs from the synapses. Serine 845 of the GluA1 subunit, a PKA substrate, is dephosphorylated during LTD, whereas S831, a CaMKII substrate, is dephosphorylated from previously potentiated synapse (Lee et al., 2000). Dephosphorylation of S845 on GluA1 is also thought to

decrease open channel probability that reduces the AMPAR mediated EPSC (Kemp and Bashir, 2001).

The phosphatase activity is mediated by protein phosphatase 1 (PP1) which is also known as Ca^{2+}/Calmodulin dependent protein phosphatase, calcineurin (Lisman, 1989; Mulkey et al., 1993). PKC activation is also required for LTD expression by triggering AMPARs internalization (Chung et al., 2000; Kim et al., 2001; Seidenman et al., 2003). PKC phosphorylation at S880 on GluA2 within the C-terminal PDZ ligand disrupts the association of GluA2 with GRIP/ABP but increases binding of PICK1, promoting endocytosis of AMPARs (Chung et al., 2000; Kim et al., 2001; Seidenman et al., 2003).

NMDAR dependent LTD in CA1 pyramidal neurons requires repeated occurrence of modest Ca^{2+} influx into the dendritic spine via NMDARs. The prolonged and weak Ca^{2+} signal is crucial for activation of protein phosphatase and calcineurin that lead to dephosphorylation of AMPARs. This drives endocytosis of AMPARs and reduces synaptic AMPARs underlying LTD expression. Thus NMDAR dependent LTD is mainly a postsynaptic mechanism by regulating synaptic trafficking of AMPARs.

mGluR-dependent LTD

The second major form of LTD in SC synapses is provided by mGluRs (Anwyl, 1999; Bolshakov and Siegelbaum, 1994). The mGluRs are enriched in the

extrasynaptic membrane and dendritic shafts, congregating in a perisynaptic ring (Baude et al., 1993; Lujan et al., 1997). A key feature of mGluR-LTD is that it is NMDAR-independent. The mGluR-mediated LTD is mechanically distinct from NMDAR-LTD and the two forms of LTD are not mutually exclusive (Oliet et al., 1997). With 3Hz/5 min stimulation, mGluR-LTD can be induced in the presence of D-AP5, an NMDAR antagonist. Conversely, NMDAR-LTD can be induced in the presence of an mGluR antagonist. Moreover, mGluR-LTD is induced more easily at hyperpolarized potentials, whereas NMDAR-LTD is favored over mGluR-LTD at depolarized potentials, indicating expression mechanisms of mGluR-LTD and NMDAR-LTD are distinct (Oilet et al., 1997).

Induction methods

The mGluR-LTD can be induced with a pairing protocol, applying repeated low-frequency presynaptic stimulation paired with postsynaptic depolarization (Kemp and Bashir, 1999; Huber et al., 2000). Paired-pulse stimulation, 900 paired stimuli at 1 Hz for 15 min, generates stable LTD in the presence of NMDAR antagonist, which is completely blocked by mGluR antagonist (Huber et al., 2000). A selective agonist for group 1 mGluRs, (S)-3,5-dihydroxyphenylglycine (DHPG), can also trigger LTD (Palmer et al., 1997). This DHPG-LTD is mainly mediated by mGluR5 in CA1 pyramidal neurons, as evidenced by complete blocking of DHPG-LTD with mGluR5 antagonists but not by mGluR1 antagonists (Faas et al., 2002).

The mGluR5 receptors play a major role in triggering mGluR-LTD at SC synapses. In mGluR5 knockout mice, DHPG-LTD is absent in CA1 pyramidal neurons (Huber et al., 2001). The mGluR1 also has a role inducing hippocampal LTD (Neyman and Manahan-Vaughan, 2008), but more clear roles of mGluR1 have been investigated in cerebellum where their expression is high (Conquet et al., 1994).

Expression of mGluR-LTD

The expression of mGluR-LTD at SC synapses involves postsynaptic mechanisms, altering AMPAR trafficking. The group 1 mGluR selective agonist, DHPG, induces LTD by rapid depletion of synaptic AMPARs in cultured hippocampal neurons and slices (Snyder et al., 2001; Xiao et al., 2001). In cultured neurons, DHPG treatment reduces the amplitude of EPSCs as well as increased internalization with reduced surface expression of AMPARs, visualized by immunocytochemistry. In addition, the amplitude of mEPSCs is reduced which can be easily explained with postsynaptic mechanisms (Snyder et al., 2001; Xiao et al., 2001).

The signaling pathway of group I mGluRs preferentially mediates PLC activation. PLC hydrolyzes phosphatidyl inositol (PIP_3) that generates inositol triphosphate (IP_3) and diacylglycerol (DAG), subsequently activating PKC. Generally, this

canonical signaling pathway of group I mGluRs causes LTD initiated by both mGluR1 and mGluR5 (Oliet et al., 1997). However, DHPG induced mGluR-LTD in Shaffer collateral to CA1 regions is Ca^{2+} independent and does not require PKC activation which means SC synapses do not utilize the typical intracellular signaling pathway of group I mGluRs (Fitzjohn et al., 2001; Schnabel et al., 1999).

The mGluR-LTD in hippocampus involves both protein kinase and protein phosphatase activity. Phosphorylation involves p38MAPKs (mitogen-activated protein kinases) (Moult et al., 2008; Bolshakov et al., 2000; Rush et al., 2002), ERK (extracellular signal-regulated kinases) (Gallagher et al., 2004), and PI3K (phosphoinositide 3-kinase) (Hou and Klann, 2004). Dephosphorylation of GluA2 by protein tyrosine phosphatases (PTP) leads to AMPARs endocytosis (Moult et al., 2008; Gladding et al., 2009; Nosyreva and Huber, 2005). Thus, mGluR-LTD in hippocampus involves different intracellular signaling cascades, protein kinases and phosphatases that are distinct from those used in NMDAR-LTD to remove synaptic AMPARs. There may be two synaptic AMPARs pool which link to different regulatory proteins, and which are regulated by different phosphorylation cascades that preserve the independence of each process (Collingridge et al., 2010).

Induction of mGluR-LTD causes new protein synthesis that is required for expression of LTD but not the induction phase (Moult et al., 2008; Huber et al.,

2000). Newly synthesized proteins include Arc/Arg 3.1 (activity-regulated cytoskeleton-associated protein) (Park et al., 2008), STEP (striatal-enriched protein phosphatase) (Zhang et al., 2008), MAP1B (microtubule associated protein 1B) (Davidkova, Carroll, 2007), and they are involved in AMPARs endocytosis upon induction of mGluR-LTD (Zhang et al., 2008; Davidkova, Carroll, 2007; Waung et al., 2008).

As discussed above, the mGluR-LTD and NMDAR-LTD utilize distinct signaling mechanisms and have differential effects leading to the induction of LTD. mGluR-LTD in hippocampus is dependent on PTP, the involvement of protein systhesis, p38MAPK, and ERK. In addition, DHPG-induced LTD is completely Ca^{2+} indepentdent as shown by that intracellular Ca^{2+} depletion by BAPTA and extracellular Ca^{2+} had no effect (Fitzjohn et al., 1999). However, they also share some common cellular mechanisms and have synergistic interactions that facilitate LTD. At resting membrane potential, mGluRs and NMDARs have a synergistic contribution to LTD when the activation of NMDARs is limited. This synergy may not be necessary when the activation of NMDARs is enhanced at more depolarized membrane potential.

Ca^{2+} activated K^+ channels

The electrical activity of excitable membranes, most notable in neurons, mediates a wide array of neuronal processes including propagation of action potentials,

neurotransmitter release, and the generation and integration of postsynaptic response. Neuronal excitability arises from established ion gradients across the intra- and extra-cellular faces of the plasma membrane. High Na^+ and Ca^{2+} concentration in extracellular fluid, and high K^+ concentration in the cell create the electro-chemical gradient. Ion channels have essential roles for changes in ion gradient by flowing Na^+ and Ca^{2+} ions inward to the cytoplasm, making membrane potentials depolarized, and K^+ ions out of cell, thus repolarizing to resting potential and stabilizing the excitable cell. The orchestrated performances of various ion channels are fundamental for electrical signal transmission of excitable cells (Hodgkin & Huxley, 1952).

In the seven decades, since K^+ current has been described (Goldman, 1943; Hodgkin & Katz, 1949), the underlying ion channels have proven to be astonishingly diverse. Comprising a superfamily of ion channels with more than 100 genes (Coetzee et al., 1999; Wei et al., 2005; Gutman et al., 2005; Goldstein et al., 2005; Kubo et al., 2005; Kim and Hoffman, 2007), K^+ channels' diversity is dramatically expanded by extensive alternative mRNA splicing and heteromeric channel assembly, thus giving rise to an arsenal of many thousands of functionally distinct K^+ channels. K^+ channels are categorized into four major families based on membrane topology, and biophysical properties: 1) inwardly rectifying K^+ (K_{ir}) channels, 2) two-pore-domain (leak) K^+ channels, 3) voltage-

gated K^+ channels (Kv channels), 4) Ca^{2+}-activated K^+ channels (Coetzee et al., 1999). Further subdivisions are based on sequence homology.

The large diversity of K^+ channels contributes to control a wide range of firing patterns, and modulate K^+ homeostasis, neurotransmitter release, hormone secretion, cell proliferation and apoptosis (Ashcroft, 2005; Jan, 2012; Stuhmer et al., 2006; Wickenden, 2002). Beyond these classical roles of K^+ channels in electrical signal processing, subsequent studies showed that activity dependent regulation and trafficking of K^+ channels serve as molecular and cellular substrate for learning and memory (Goldstein et al., 2001; Stackman et al., 2002; Zhang and Linden, 2003; Lin et al., 2008; Kim and Hoffman, 2008; Ganguly and Poo, 2013; Humphries and Dart, 2015). Since my thesis work stems from Ca^{2+} activated K^+ channels, it is worthwhile to present a short review.

Overview

Ca^{2+} is a ubiquitous 2nd messenger and changes in the intracellular Ca^{2+} concentration regulate a plethora of signaling transduction pathways that can impact virtually every cellular function. One mechanism through which Ca^{2+} transients influence excitability is through Ca^{2+} activated K^+ channels. Ca^{2+} activated K^+ channels have evolved to utilize this 2nd messenger as a means of providing negative feedback regulation of Ca^{2+} influx and neuronal excitability.

The first indication of the existence of Ca^{2+} activated K^+ channels was discovering increases in K^+ permeability in response to increased intracellular Ca^{2+} level in red blood cells, where it gave rise to membrane hyperpolarization and cell shrinkage (Gardos, 1958). Subsequently, the first intracellular electrophysiological recordings measured Ca^{2+} activated K^+ current (K_{Ca}) in molluscan neurons (Meech and Strumwasser, 1970) and cat spinal motor neurons (Krnjevic and Lisiewicz, 1972). Subsequent studies have shown that K_{Ca} channel activity contributes to the afterhypolarization (AHP) that follows bursts of action potentials, which regulates membrane excitability and spike frequency adaptation in many neurons (Lancaster and Adams, 1986; Alger and Nicoll, 1980; Hotson and Prince, 1980; Schwartzkroin and Stafstrom, 1980; Shao et al., 1999; Sah and Faber, 2002).

In mammalian neurons, two major families of K_{Ca} channels have been identified and can be distinguished based on, primary amino acid sequence, biophysical and pharmacological properties. One, displaying large unitary conductance and gated by associative action of membrane voltage and intracellular Ca^{2+} level (Marty, 1981; Pallotta et al., 1981; Latorre et al., 1982; McManus, 1991), is referred to as big-conductance (200-400 pS) K^+ channels (BK/K_{Ca} 1.1), and the other, exhibiting small unitary conductance and gated solely by intracellular Ca^{2+} level, is termed small-conductance (10 pS) K^+ channels (SK/K_{Ca} 2.1, K_{Ca} 2.2, K_{Ca} 2.3) and intermediate-conductance (40 pS) K^+ channels (IK/K_{Ca} 3.1).

BK channels

BK channels are referred to as MaxiK due to their large unitary K^+ conductance of greater than 200 pS, and their open probability is governed by the interplay of increased intracellular Ca^{2+} level and membrane depolarization (Marty, 1981; Pallotta et al., 1981; Latorre et al., 1982; McManus, 1991). They were the first K_{Ca} channel to be described with single channel recording in chromaffin cells, cultured rat skeletal muscle, and rabbit transverse tubules (Marty, 1981; Pallotta et al., 1981; Latorre et al., 1982). BK channels were the first of the K_{Ca} channels to be cloned from *Drosophilia*. In this case, the *slowpoke* gene product was shown to encode functional BK channels (Atkinson et al., 1991; Adelman et al., 1992). The mammalian orthologue of BK channels was cloned a short time later from mouse and a number of other species (Tseng-Crank et al., 1994; Pallanck et al., 1994). In addition, alternative mRNA splicing, particularly in the intracellular C-terminal domain accomplishes remarkable functional diversity of BK channels, potentially encoding more than 1000 different BK channel subtypes (Lagrutta et al., 1994).

Molecular structure of BK channels

Functional BK channels are tetrameric structures, consisting of four pore-forming α-subunits (Shen et al., 1994), which can coassemble with modulatory β-subunits (Adelman et al., 1992; McManus et al., 1995). Each α-subunit contains seven transmembrane domains (S0 – S6) with an extensive intracellular C-terminus (S7

– S10) (Meera et al., 1997). The intracellular C-terminus of the α-subunit (S7 – S10) comprises approximately 70% of its total length (Wei et al., 1994; Meera et al., 1997). Functionally, the α-subunit has three distinct structural domains that can work as independent modules. The voltage sensor domain (S0 – S4) senses changes in membrane potential, S5 and S6 contribute the pore-forming region and control ion selectivity and permeation. The cytoplasmic tail domain (S7 – S10) forms the gating-ring and serves as the intracellular Ca^{2+} sensor and the binding sites for other ligands to influence the open probability of the channel.

The primary structure of the α-subunit transmembrane domain shows significant homology to the transmembrane segments (S1 – S6) of the voltage-gated K^+ channels, particularly in the voltage sensor domain and pore-forming regions (Jan and Jan, 1997). In addition, the homologous functional properties of Kv channels, such as selective permeation of K^+ ion and voltage dependence of gating process, also can be found in BK channels (Heginbotham et al., 1994; Cui et al., 1997).

BK channels show a high degree of selectivity for K^+ ions. This capability of selective ion permeation is conferred by the conserved sequence “GYG” in the pore-forming region, which serves as the K^+ selectivity filter that excludes anions, Na^+, Ca^{2+}, and Mg^{2+} but selectively allows K^+ ions to go through (Heginbotham et al., 1994; Doyle et al., 1998). One thing to note is that despite such a selective

ion permeation of BK channels, BK channels produce the largest single unitary conductance of any K^+ channel. The large conductance is partly derived from clusters of negatively charged amino acids localized at both intracellular and extracellular entrances of the central permeation pathway. These residues form rings of negative charges that serve as an electrostatic trap to increase the local K^+ concentration at the extracellular face of the channel (Brelidze et al., 2003; Nimigean et al., 2003). Additionally, the inner vestibule has a broad entrance with an enlarged cavity and inner pore are thought to further contribute to large conductance of these channels (Li et al., 2004; Brelidze et al., 2005).

The gating sensor for membrane potential

Membrane depolarization, albeit to extreme positive potentials, is sufficient for the activation of BK channels (Cui et al., 1997). Voltage dependence of BK channels stems from voltage sensing residues in the S4 segment. Positively charged residues occupy every third position in the voltage sensors. Membrane depolarization triggers the rearrangement of charged residues and generates gating current even at lower Ca^{2+} concentrations (Cui et al., 1997). Thus, BK channels are purely voltage dependent and membrane depolarization is sufficient to activate BK channels in the absence of Ca^{2+} (Cui et al., 1997).

However, the voltage sensor domain of BK channels displays distinct functional features from that of Kv channels. BK channels exhibit lower gating charge compared to Kv channels, resulting in weaker voltage sensitivity (Stefani et al.,

1997; Horrigan and Aldrich, 1999; Ma et al., 2006). BK channels carry 2.4*e* charges per channels whereas the canonical Shaker K^+ channels exhibit 12~13*e* gating charges, suggesting more membrane depolarization is required for BK channels to reach fully activated state (Horrigan and Aldrich, 1999). This weaker voltage sensitivity has the physiological benefit conferring BK channels to be responsive to a broad range of membrane potentials to fine-tune its activation. (Yang et al., 2015).

Ca^{2+} sensor and gating mechanism

BK channels gating is modulated by intracellular Ca^{2+} ions with the K_D ~10 uM. BK channels have two Ca^{2+} binding domains in the cytosolic C-tail domain (S7-S10). High affinity Ca^{2+} binding domains are embedded in two regulators of conductance for K^+, RCK1 and RCK2, which are connected by an approximately 100 amino acid long linker sequence. Within the tetrameric structure of BK channels of each cytoplasmic C-tail, each tandem RCK1-RCK2 stacks and interlocks together, forming a large gating ring-structure (Wu et al., 2010; Yuan et al., 2010). The C-terminus of RCK2 domain includes high affinity Ca^{2+} sensing domain termed as "Ca^{2+} bowl", consisting of a string of aspartic acid residues (Schreiber and Salkoff, 1997). Mutations within the Ca^{2+} bowl region cause decreased Ca^{2+} sensitivity and shifts in the conductance-voltage (G-V) curve to more depolarized membrane potentials (Schreiber et al., 1997). The Ca^{2+} bowl in RCK2 forms an E-F-hand-like motif at the assembly interface between two

subunits, which is critical for Ca^{2+} sensitivity (Jiang et al., 2002; Yuan et al., 2010; Wu et al., 2010). Furthermore, Ca^{2+} binding to RCK2 has been shown to induce conformational rearrangements that underlie the Ca^{2+} gating mechanism of BK channels (Yusifov et al., 2008). The other high affinity Ca^{2+} sensor is located in RCK1 domain characterized by D362/D367 and M513 residues (Xia et al., 2002; Bao et al., 2002; Zeng et al., 2005). Mutational study has shown that these residues are critical to Ca^{2+} sensing for activation, and influence the kinetics of activation and deactivation, although clear diffraction of Ca^{2+} ions binding within RCK1 domain is not resolved yet (Yuan et al., 2010).

Pharmacology of BK channels

Numerous pharmacological modulators have been described for BK channels. The K^+ channels blocker tetraethylammonium (TEA) can block BK channels in the micromolar range (Blatz et al., 1987). The sensitivity of TEA is due to the phenylalanine ring in the boundaries of the external mouth of the selectivity filter (Lagrutta et al., 1998; Heginbotham et al., 1992). Scorpion-derived peptides charybodotoxin and iberiotoxin act as pore blockers, occluding the conduction pathways of the α subunit in BK channels (Miller et al., 1985; Candia et al., 1992). Kaliotoxin is another potent blocker isolated from scorpion, blocking BK channel opening with a K_D of close to 20 nM (Crest et al., 1992). Mycotoxin paxilline and penitrem A have been described as potent BK channel blockers with a K_D in nanomolar range (Knaus et al., 1994; Sanchez et al., 1996; Strobaek

et al., 2000). Among those agents, iberitoxin and paxilline are highly selective for BK channels whereas other agents block numerous other K^+ channels. Furthermore, numerous small molecule openers and activators of BK channels have been identified. NS1619, synthetic benzimidazolone derivative, and the natural modulator, dehydrosoyasaponin-1 (DHS-1), isolated from a Ghanese medicinal herb, specifically activate BK channels to open (Gribkoff et al., 1996). The compound NS11021 has been shown to have higher selectivity and 10 times higher potency than NS1619 (Bentzen et al., 2007).

β-subunits

Four types of accessory β-subunit to BK channels have been found in mammalian (Knaus et al., 1994; Brenner et al., 2000) and associate with the α-subunit, forming heteromeric complexes of BK channels. β1, β2, and β3 exhibit a high degree of sequence homology whereas β4 is distant from the other β-subunits. All four BK β-subunits share a membrane topology with two transmembrane segments with short intracellular N- and C-terminal regions (Knaus et al., 1994; Orio et al., 2002; Torres et al., 2007).

Coassembly of α- and β-subunit results in enhanced Ca^{2+}, voltage sensitivity, and gating properties of BK channels that influences activation/deactivation kinetics of channels. Coexpression of β1 subunits with the α subunit leads to the channel opening at more hyperpolarized potential and sustained non-inactivating

current (Bao et al., 2005), which is mediated by the interaction of β1 with S0 domain of α-subunit (Orio et al., 2006; Wallner et al., 1999). Association with β4 subunit leads to slower activation/deactivation kinetics (Ha et al., 2004; Weiger et al., 2000). The β2/3 subunits endow channels with rapid inactivation through blockade of channels by the N-terminus cytoplasmic inactivation "ball" (Solaro et al., 1997; Wallner et al., 1999; Xia et al., 1999; Brenner et al., 2000). In addition, coexpression of α and β subunit alters the pharmacology of BK channels. The affinity of scorpion toxin and DHS-1 to BK channels is increased by the β1 subunit (Dworetzky et al., 1996; McManus et al., 1995). In contrast, coexpression of β2/3 reduces channel's sensitivity to charybdotoxin and DHS-1 (Ding et al., 1998; Wallner et al., 1999; Xia et al., 1999), and β4 subunit leads to insensitivity to iberiotoxin and charybdotoxin (Meera et al., 2000).

Physiological roles of BK channels

BK channels are expressed broadly in smooth and skeletal muscle, and neural tissue in brain, which serve as a negative feedback of Ca^{2+} entry by linking cellular Ca^{2+} homeostasis and excitability (Brayden and Nelson, 1992; Pluger et al., 2000; Adams et al., 1982; Storm, 1987). Owing to their large conductance, the opening of BK channels leads to efflux of K^+ current which effectively hyperpolarizes the cell and limits Ca^{2+} entry via voltage-gated Ca^{2+} channels. Thus, BK channels regulate cellular excitability in a variety of physiological processes, including in smooth muscle tone (Brayden et al., 2003; Meredith et

al., 2004), microbial killing in leukocytes (Ahluwalia et al., 2004), hormone secretion (Waring et al., 2009) and neurotransmitter release (Robitaille et al., 1993; Raffaelli et al., 2004). In the central nervous system, BK channels are highly expressed in terminal area of fiber tract; axon, presynaptic terminal, and cell body (Knaus et al., 1996; Hu et al., 2001; Sailer et al., 2006; Kaufmann et al., 2006). In SC synapses, presynaptic BK channels in CA3 cells are activated by Ca^{2+} influx and contribute to repolarization of presynaptic action potential and transmitter release (Hu et al., 2001). In CA1 pyramidal neurons, BK channels generate macroscopic currents and contribute to repolarize action potentials (Storm, 1987), mediate the fast phase of afterhyperpolarization (fAHP) following an action potential (Lancaster and Nicoll, 1987; Gu et al., 2007), which influences repetitive firing (Shao et al., 1999; Gu et al., 2007). BK channels also shape the dendritic Ca^{2+} spike in CA1 pyramidal neurons (Golding et al., 1999). In the cochlea hair cells, BK channels are activated by Ca^{2+} influx through L-type Ca^{2+} channels and influence the oscillation in membrane potential (Roberts et al., 1990). In the cerebellum, BK channels are abundant in the deep cerebellar nuclei as well as on the soma and dendritic trunk of Purkinje cells, and contribute to the generation of the AHP (Womack et al., 2002).

SK channels

Four homologous genes, SK1-4 (KCNN1-4) encode SK channels. SK1-3 subunits were first identified and cloned from mammalian brain (Kohler et al.,

1996). They have small single-channel conductances of ~10 pS while SK4 (IK1) has a conductance of ~40 pS. All members of the family are solely gated by rises in intracellular Ca^{2+} within the submicromolar range. SK1-3 channels are expressed throughout the CNS in distinct and overlapping patterns (Stocker, 2000). The existence of SK channels was revealed by the neurotoxin apamin, which is a component of honey bee venom. Apamin is an 18-amino-acid peptide with two disulfide bridges and a C-terminal amide. In cultured rat skeletal muscle, SK mediated Ca^{2+} activated K^+ currents are blocked by apamin but not by 5 mM external TEA. This apamin sensitive SK current is highly Ca^{2+} sensitive and associated with afterhyperpolarization (Blatz, Magleby, 1986). Apamin is by far the most selective SK channel blocker, and there are no known off targets after more than 70 years of study. Moreover, apamin readily crosses the blood-brain barrier. Thus, the consequences of apamin application can be ascribed to blockade of SK channels.

SK4/IK1 is highly expressed in the peripheral tissues, particularly smooth muscle tissues (Ishii et al., 1997) with some expression in brain (Allen Brain Atlas). IK1 channels exhibit voltage independence and Ca^{2+} activated K^+ permeation as other SK family members (Ishii et al., 1997). IK channels present a distinct pharmacological profile that is insensitive to apamin and iberiotoxin but blocked by charybdotoxin, clotrimazole, and more selectively by the clotrimazole derivative TRAM-34 (Joiner et al., 1997; Logsdon et al., 1997; Ishii et al., 1997; Wulff et al., 2000). This biophysical and pharmacological profile of heterologously

expressed IK channels corresponds to the native Gardos channels in red blood cells (Gardos, 1958; Ishii et al., 1997).

SK channels cloning and biophysics

The transmembrane structure of SK channels shares overall topology with members of voltage-gated potassium channel superfamily, presenting six transmembrane (TM) spanning regions (S1 – S6) with intracellular N- and C-termini and S5 and S6 comprise the pore-forming region (Kohler et al., 1996). SK channels may be homomeric tetramers, or heteromeric assemblies of different SK subunits both in heterologous expression systems (Monaghan et al., 2004) and in native tissue (Strassmaier et al., 2005). The sequence analysis shows that SK channels also share homology with all other potassium channels in the transmembrane core region between S5 and S6 domain where the pore loop is formed (Kohler et al., 1996). In contrast to these structural similarities with Kv channels, SK channels contain only a few positively charged residues in the S4 segment and there is no voltage dependence to their gating process (Blatz and MAgleby, 1986; Hirschberg and Maylie et al., 1998). In addition, there is less degree of sequence conservation in cytosolic N-and C-termini where multiple phosphorylation sites are placed which influence physiological properties of SK channels.

Notably, among SK subunits, the most conserved domain is found, not in the TM regions of the subunits but in the proximal C-terminal domain that is responsible for Ca^{2+} gating; this is discussed below.

SK channels gating

Gating by intracellular Ca^{2+} is the key feature of SK channels. Macroscopic and single channel recordings show that all SK subtypes reveal similar Ca^{2+} gating profiles. They have rapid response to Ca^{2+} in 5-15 ms range of time constant with half maximal activation (EC_{50}) of ~0.5 uM in a voltage independent manner. Ca^{2+} gating has a Hill coefficient close to 4, suggesting binding of at least four Ca^{2+} ions is required for activation and Ca^{2+} binding is cooperative (Kohler et al., 1996; Hirschberg et al, 1998; Xia et al., 1998).

In fact, SK channels do not contain apparent Ca^{2+} binding motifs such as E-F hand, C2A motif, or Ca^{2+} bowl sequence that may mediate Ca^{2+} binding. Rather the Ca^{2+} gating of SK channels is endowed by the constitutive binding of calmodulin (CaM) to the CaM-binding domain (CaMBD) in the proximal portion of the intracellular C-terminal domain (Fig. 1). CaM contains two E-F hand motifs within both N- and C-globular regions, separated by a flexible linker. The C-terminal E-F hands in CaM interact with CaMBD in Ca^{2+} independent manner, whereas Ca^{2+} binding to the N-lobe of E-F hands of CaM induces gating (Xia et al., 1998; Keen et al., 1999). The defining experiment that demonstrated how

CaM acts as the SK channel gating trigger was to express SK2 channels in *Xenopus* oocytes together with CaM harboring mutations that abrogated E-F hand Ca^{2+} binding. With only one N-terminal E-F hand intact, the Ca2+ sensitivity of the SK2 current was dramatically reduced and the Hill coefficient shifted from ~4 to ~2 (Xia et al., 1998) Structural analyses showed that CaM-CaMBD complex is monomeric in the absence of Ca^{2+}. Once Ca^{2+} binds to the N-terminal lobe of CaM, significant conformational rearrangement occurs in CaM that allows a contact with the distal CaMBD on a neighboring subunit for dimerization. When both CaM-CaMBD complexes are dimerized, forming a dimer of dimers, this rearrangement results in rotatory movement of S6 pore helices, subsequently transmiting mechanical force to open the SK channel gate (Xia et al., 1998; Keen et al., 1999; Schumacher et al., 2001).

The gating of SK channels by Ca^{2+} is modulated by protein kinase CK2 and protein phosphatase 2A (PP2A), which are also constitutively bound to SK channels (Fig. 1) (Bildl et al., 2004; Allen et al., 2007). CK2 phosphorylates SK bound CaM at T80, but not the channel itself. The CK2 phosphorylation of CaM reduces Ca^{2+} sensitivity of SK channels by ~5 fold, and increases the deactivation rate by ~4 fold. This phosphorylation of CaM is state dependent that only occurs when SK channel is closed, which is reversed by PP2A. When SK channels are open, PP2A dephosphorylates SK2 bound CaM at T80 that increases Ca^{2+} sensitivity of SK channels, in turn regulating open channel

probability. This state dependent feedback mechanism by CK2 and PP2A contributes fine-tuning of SK2 channels to adjust local Ca^{2+} level in activity dependent manner (Allen et al., 2007).

Despite the fact that the activation of SK channels is voltage independent, SK channels exhibit inward rectification (Kohler et al, 1996; Xia et al, 1998). This property was ascribed to a voltage-dependent block of SK channels by intracellular divalent cations (Soh et al, 2001). At positive membrane potentials, Ca^{2+}, Sr^{2+}, and Ba^{2+} ions not only activate but also block SK channels in a concentration-dependent manner. However, a more recent study shows that this inward rectification is an intrinsic property of SK channels (Li et al., 2011). They identified three charged residues in the S6 TM region near the inner mouth of the pore that regulates the conductance of rectification by an electrostatic effect on the entry rate of K^+ ions into the pore. Moreover, these charged residues also influence Ca^{2+} affinity and open probability of the channel in the absence of Ca^{2+}.

Pharmacology of SK channels

SK channels subtypes can be distinguished on the basis of apamin sensitivity: SK1 (least sensitive, EC_{50} ~ 10 nM), SK2 (most sensitive, EC_{50} ~ 40 pM), SK3 (intermediate-sensitive, EC_{50} ~ 1 nM) (Ishii et al., 1997; Shah, Haylett, 2000). Several amino acid residues in the outer vestibule region are essential for apamin sensitivity. Mutations in the outer pore region of SK1 and SK3

significantly increased sensitivity to block by apamin. Further, chimeric subunits revealed that amino acids outside the pore are involved in apamin binding (Ishii et al., 1997; D'Hoedt et al., 2004), particularly within the S3-S4 extracellular loop (Nolting et al., 2007). One thing to note is that binding affinity for apamin (K_D) and potency of block (I_{50}) displays significant differences suggesting that a simple one-to-one binding model does not reveal the mechanism of SK channel-apamin interaction (Finlayson et al., 2001; Grunnet et al., 2001a,b). This suggests that apamin does not act just as a pore blocker, but binding and inhibiting SK channel activity are separate processes, rather apamin acts as an allosteric modulator (Lamy et al., 2010). Molecular modeling suggests that apamin binds to residues in the S3-S4 extracellular loop and outer pore region of S5-S6. Apamin binding may disrupt local bonding between S3-S4 extracellular loop and the outer pore region of the channels, which can lead to structural collapse of the selectivity filter, resuling in a non-conducting state (Weatherall et al., 2010).

Beside apamin, other peptides and organic compounds, tubocurarine, quaternary salts of bicuculline, dequalinium, curare, UCL 1648 and UCL 1848 also block SK channels with non-subtype selectivity (Johnson et al., 1997; Seutin et al., 1999; Liegeois et al., 2003). Other toxins isolated from scorpion venom preferentially block SK2 subunit, including tamapin, isolated from *Mesobuthus tamulus* (Pedarzani et al., 2002) and scyllatoxin, isolated from the scorpion *Leiurus quinquestriatus* (Castle et al., 1986). Furthermore, some compounds have been

shown to enhance the activity of SK channels. These include 1-ethyl-2-benzimIdazolinone (1-EBIO) and 6,7-dichloro-1H-indole-2,3-dione 3-oxime (NS309), non-selective positive modulator (Strøbaek et al., 2004). N-Cyclohexyl-N-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-4-pyrimidinamine (CyPPA) is selective positive modulator of SK2 and SK3 (Hougaard et al., 2007).

Distribution and localization of SK subunits

SK channels are predominantly expressed throughout the mammalian central nervous system. Northern blot analysis and in situ hybridization, in accordance with immunohistochemistry, have shown that SK1, SK2, SK3 subunits are partially overlapping but have distinct expression patterns; SK1 and SK2 showing extensive co-localization, and SK3 presenting a complementary distribution (Stocker and Pedarzani, 2000; Tacconi et al., 2001; Sailer et al., 2004). The SK1 and SK2 subunits are co-expressed at their high density in the hippocampus, neocortex, thalamus, and in cranial motor neuron (Tacconi et al., 2001; Sailer et al., 2004; Stocker et al., 1999), whereas SK3 subunit are maximally expressed in thalamus, hypothalamus, lateral septum, medial habenula, supraoptic nucleus, several brain stem nuclei, and in many monoaminergic neurons in midbrain (Tacconi et al., 2001; Sailer et al., 2004; Wolfart et al., 2001). This suggests the role of specific SK subunit in distinct brain regions on cellular level, and possibly in different subcellular compartments.

Diversity of SK channels

In addition to their ability to form heteromeric channels, further diversity in SK channels can be accomplished by alternative mRNA splicing. In particular, the SK1 mRNA may be alternatively spliced, yielding up to 32 disintct transcripts, and at least 20 transcripts have been detected in the mouse brain (Shmukler et al., 2001). This alternative splicing is not only limited to SK1 gene. Further study revealed SK3 variants in human brain resulting in polypeptides with amino terminal truncations (Tomita et al., 2003; Kolski-Andreaco et al., 2004) or small insertion between S5 and pore region behaving as a dominant negative to SK channels and lacking apamin sensitivity (Wittekindt et al., 2004). In addition, two isoforms of SK2 proteins have been described that differ in the length of the N-terminal domain (Strassmaier et al., 2005). Long SK2 (SK2-L) isoform has an additional 207 amino acids at N-terminus compared to the short SK2 (SK2-S) and they are produced from separate promoters (Fig. 2). SK2-L and SK2-S are co-expressed throughout the brain and co-immunoprecipitate from brain and heterologous coexpression system (Strassmaier et al., 2005). This unique N-terminus SK2-L is crucial for synaptic localization and function of SK2-containing channels (Allen et al., 2011). The functional role of SK2-L will be discussed later.

Physiological roles of SK channels

In central nervous systems, SK channels have fundamental roles in controlling neuronal excitability by providing feedback regulation of Ca^{2+} dependent

processes. The most studied example for this type of feedback is their contribution to the AHP that follows action potentials. The AHP influences firing frequency that ultimately contributes to spike frequency adaptation and neuronal integration. The AHP in many neurons comprises three overlapping kinetics components; fast (fAHP), medium (mAHP), and slow (sAHP) (Sah, 1996). The fAHP activates rapidly and typically lasts for 10-20 ms, and, in some cases such as CA1 pyramidal neurons is mediated by BK channels and some voltage gated K^+ channels which is responsible for action potential repolarization (Storm, 1987; Sah et al., 2002; Gu et al., 2007). The mAHP also activates rapidly and lasts approximately 100 ms. In many cases, but not CA1 pyramidal neurons, the mAHP is predominantly blocked by apamin, selective blocker of SK channels (Stocker, 1999). BK channels and KCNQ also contribute to the mAHP depending on neuronal subtype (Storm, 1989). The apamin sensitive mAHP has demonstrated to contribute to the instantaneous firing rate in layer V sensorimotor cortical neurons (Schwindt et al., 1988), to setting the tonic firing frequency in dorsal vagal neurons (Sah et al., 1992), to regulating burst firing and rhythmic oscillatory activity in midbrain dopaminergic neurons (Seutin et al., 1993), in nucleus reticularis thalamic neuron (Bal et al., 1993), in inferior olive neurons (Bal et al., 1997), and in hypothalamic supraoptic neurons (Hu et al., 1992), cerebellar Purkinje neurons (Womack et al., 2003). The sAHP typically lasts several seconds, and it is Ca^{2+} dependent, voltage independent, K^+ selective, and plays a major role in controlling spike frequency adaptation. The

slow AHP channels are apamin insensitive and their molecular identity is not known.

The contribution of SK channels to AHP is largely due to Ca^{2+} entry through VGCCs. Different subtypes of VGCCs are coupled to SK channels in a cell type specific manner. In subthalamic nucleus neurons, N type Ca^{2+} channels (Cav2.2) are selectively coupled to SK channels that influence precision and the pattern of action potentials (Hallworth et al., 2003). P/Q type Ca^{2+} channels provide the Ca^{2+} source for SK channels in Purkinje neurons whereas in their downstream target in the deep cerebellar nucleus, SK currents are activated by Ca^{2+} influx through T type Ca^{2+} channels (Edgerton and Reinhart, 2003; Alviña et al., 2009). In midbrain dopaminergic neurons, T type Ca^{2+} channels are functionally coupled to SK channels (Wolfart and Roeper, 2002). L and R type Ca^{2+} channels provide the Ca^{2+} to activate SK channels in suprachiasmatic nucleus neurons (Cloues and Sather, 2003). T type Ca^{2+} channels provides Ca^{2+} source for SK2 channel–dependent AHPs in dendrites of nucleus reticularis thalami (Cueni et al., 2008).

The apamin sensitive SK currents have been measured in voltage clamp recordings from CA1 pyramidal neurons (Fig. 3) (Bond et al., 2004). This apamin sensitive current in CA1 pyramidal neurons is due to SK2-containing channels (Bond et al., 2004). However, SK channel activity does not contribute to the AHP in CA1 pyramidal neurons (Gu et al., 2004).

SK channels in CA1 pyramidal neuron

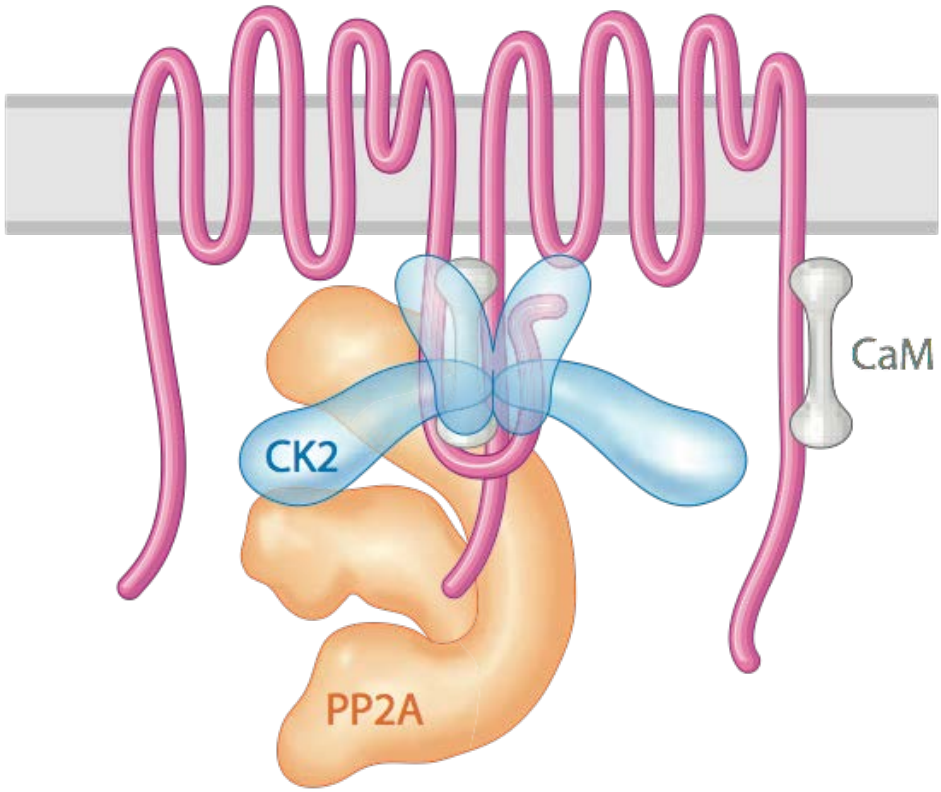
SK2-containing channels are expressed throughout the dendritic arbor of CA1 pyramidal neurons and in the PSD of spines (Lin et al., 2008; Ballesteros-Merino et al., 2012). Immuno-gold EM labeling revealed colocalization of SK2 and NR1 specific gold particles in the PSD (Lin et al., 2008) and SK2 channels are functionally coupled to NMDARs as a synaptically activated Ca^{2+} source (Fig. 4) (Ngo-Anh et al., 2005). Electrophysiological whole cell recording showed that blocking NMDARs with AP5 occludes apamin sensitivity on EPSPs, demonstrating SK channel activity requires the activation of NMDARs (Ngo-Anh et al., 2005). Evoked synaptic stimulation activates AMPARs that depolarize the postsynaptic spine, resulting in Mg^{2+} unblock of NMDARs. Activation of NMDARs leads to Ca^{2+} influx into the stimulated dendritic spine. SK2 channels, forming a nanodomain with NMDARs in the PSD of the dendritic spine, are activated by this intracellular Ca^{2+} and provide an outward repolarizing K^{+} conductance that reduces EPSPs, favors reblocking of NMDARs, and reduces the spine Ca^{2+} transient (Ngo-Anh et al., 2005; Lin et al., 2008). Upon the induction of LTP, PKA and CaMKII phosphorylate GluA1-containing AMPARs that increases trafficking of AMPARs into the PSD (Shi et al., 1999; Hayashi et al., 2000; Makino et al., 2009) whereas LTP induction removes SK2-containing channels from PSD (Lin et al., 2008). The synaptic removal of SK2-containing channels after LTP requires the activation of NMDARs and PKA. The application of blockers for NMDAR and PKA prevents the endocytosis of synaptic SK2 after LTP induction

as shown by the existence of apamin effect on EPSP. This was further supported by immuno-EM, revealing substantial amount of immunoparticles of SK2 in PSD after LTP induction with PKA blocker treatment (Lin et al., 2008). This PKA dependent endocytosis of SK2 channels from the PSD requires precedent synaptic translocation of GluA1-containing AMPA receptors into the synapse (Lin et al., 2010). This is supported by prevention of SK2 endocytosis with the blocking exocytosis of AMPARs with partial peptide of GluA1 C-terminal with PDZ ligand after LTP induction. However, inhibiting of SK2 endocytosis by adding partial peptide of $SK2_{563-576}$, which includes the substrate of PKA phosphorylation, does not block AMPARs exocytosis during LTP (Lin et al., 2008; Lin et al., 2010). This suggests that synaptic trafficking of AMPARs procedes SK2 endocytosis that contribute majority of expression of LTP.

The modulation of SK channel activity influences synaptic plasticity, which is cellular substrate for learning and memory. Field recordings in area CA1 showed that blocking SK2 channels with apamin facilitates the induction of synaptic plasticity (Stackman et al., 2002). SK channel blockade by apamin treatment shifted the threshold for induction of NMDA dependent synaptic plasticity to lower stimulus frequencies, and enhanced hippocampus dependent learning and memory encoding (Stackman et al., 2002). In contrast, transgenic overexpression of SK2 channels impairs the induction of synaptic plasticity and hippocampus dependent learning (Hammond et al., 2006; Stackman et al., 2008).

SK2-L and SK2-S can form functional homomeric channels with similar Ca^{2+} sensitivity and the amplitude of whole-cell SK2 current when expressed in cell lines, although the amplitude of SK2-L current in excised patch is much smaller than that of SK2-S (Strassmaier et al., 2005). This study also showed that SK2-L leads to punctate surface expression compared to more diffuse expression of SK2-S as evidenced by immunocytochemistry. Indeed, SK2-L in CA1 pyramidal neurons directs subcellular localization of native SK2-containing channels into the PSD (Allen et al., 2011). SK2-S only expressing mice, lacking SK2-L isoform, have no apamin response in their EPSPs and show disrupted synaptic distribution of SK2 channels evidenced by immuno-EM. However, re-expressing SK2-L in SK2-S only mice re-establishes synaptic SK2 localization and function. Thus, the SK2-L isoform is necessary for synaptic localization and function of SK2-containing channels in CA1 pyramidal neurons. The main question in this study is how the unique 207 amino acids at the N-terminus of SK2-L endow the synaptic localization, and what is the molecular mechanism for the synaptic recruitment. My thesis work directly addresses these questions.

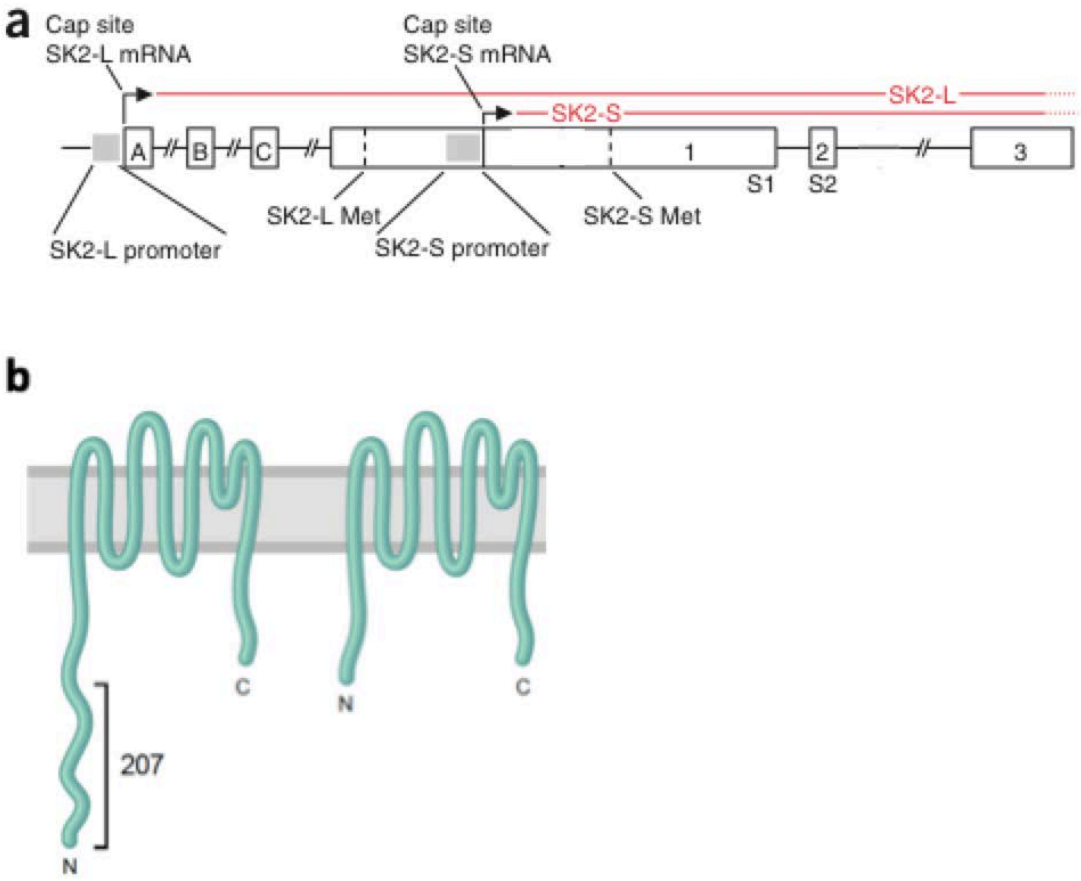
Figure 1. Structure and modulation of SK channels.



(Allen et al., 2007)

Figure 1. SK channels are multiprotein complexes, composed of tetrameric assemblies of alpha subunits and modulatory proteins. CaM constitutively associates with SK channels and confers Ca^{2+} gating. CK2 and PP2A coassemble with SK channels and determine phosphorylation status of SK-bound CaM which regulates Ca^{2+} gating. CK2 phosphorylates SK-bound CaM in closed state and reduces channel activity, whereas PP2A dephosphorylates CaM and has an opposing role in Ca^{2+} gating.

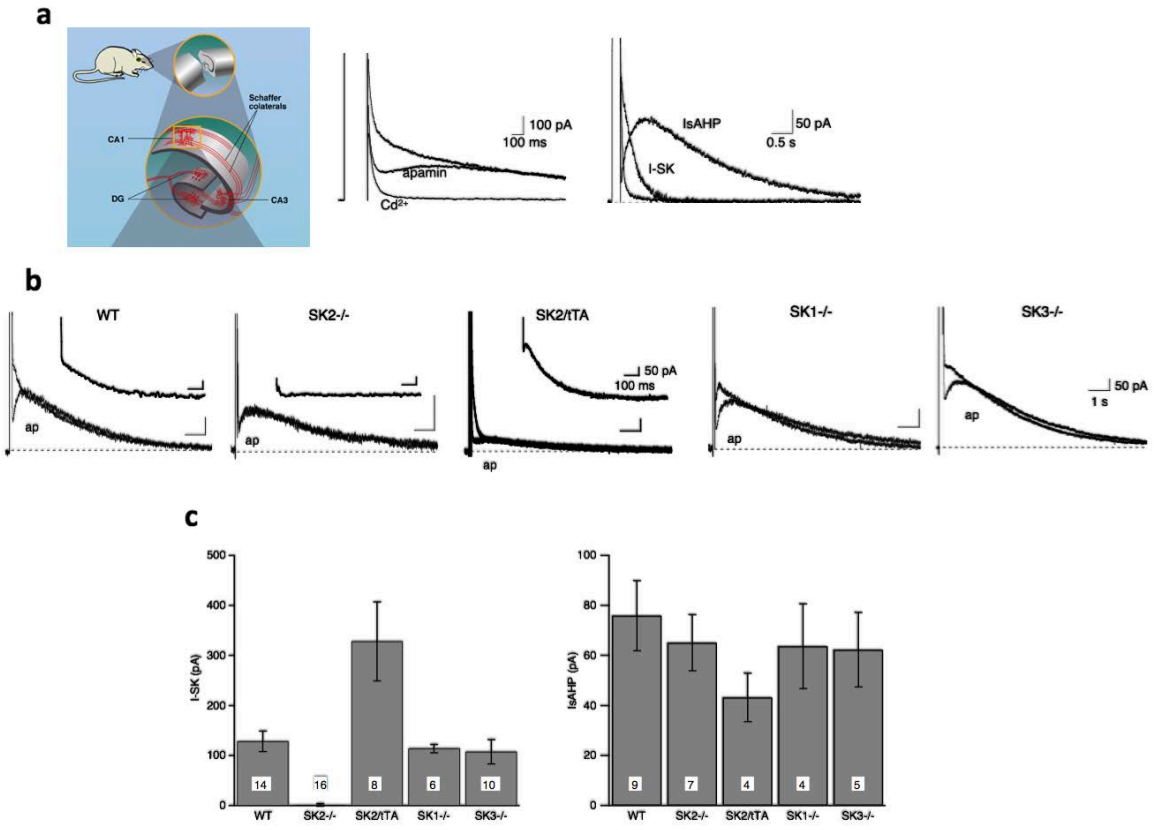
Figure 2. Two isoforms of SK2.



(Allen et al., 2011)

Figure 2. The SK2 gene encodes two isoforms of SK2. (**a**) The 5' region of the mouse SK2 gene. Boxes represent exons. Darker area represents the position of SK2-L and SK2-S promoters. The red line indicates primary transcripts of for SK2-L and SK2-S. The cap site for SK2-L resides in exon A, whereas the 5' end of SK2-S resides in exon 1. The translational initiator methionine codons for SK2-L and SK2-S is shown as dotted line. (**b**) SK2-L contains an additional 207 N-terminal amino acids compared to SK2-S.

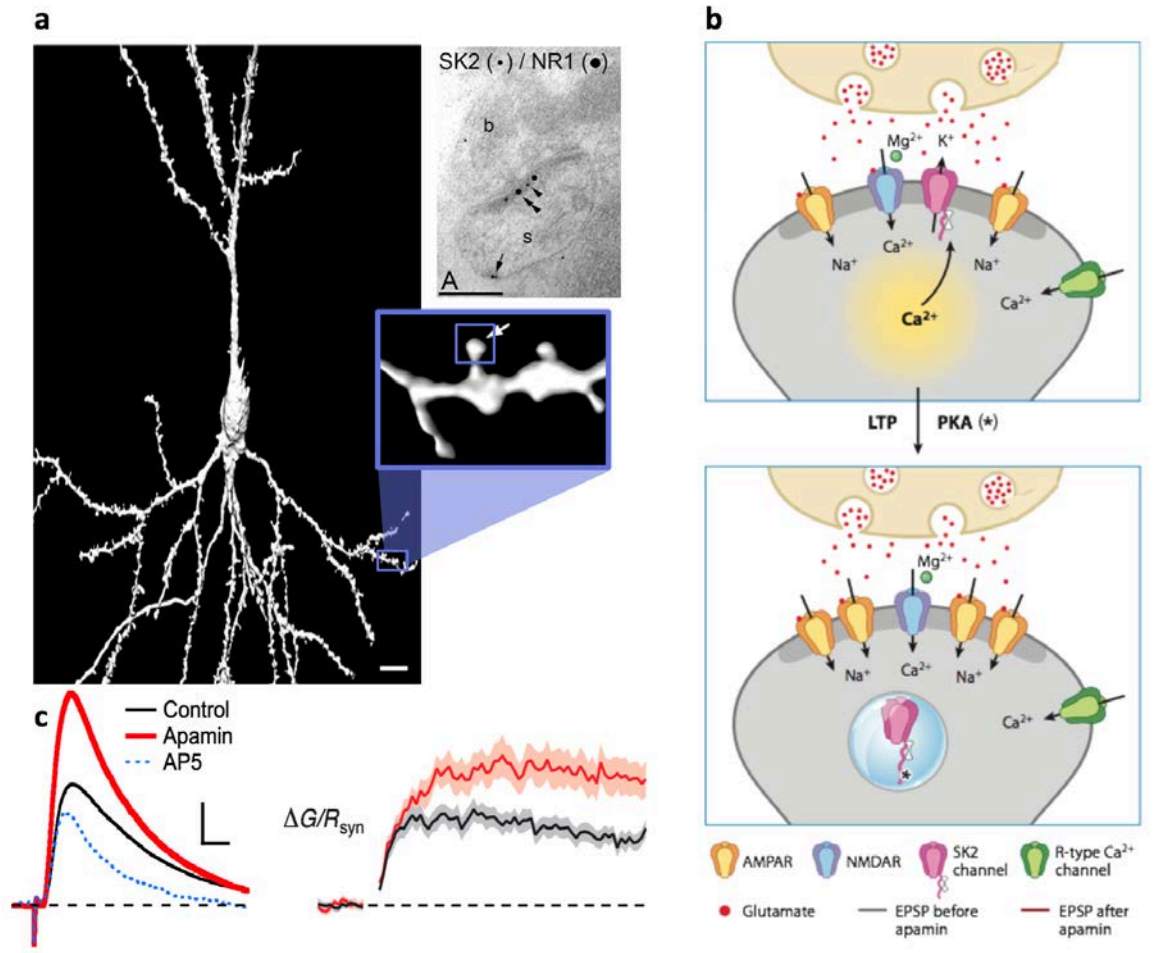
Figure 3. SK currents in CA1 pyramidal neurons.



(Bond et al., 2004)

Figure 3. SK2 channels underlie the apamin sensitive currents in hippocampal CA1 pyramidal neurons. (**a**) In voltage clamp configurations of CA1 neurons, tail currents are elicited by 100 ms depolarizing pulse to +20 mV followed by a return to the –55 mV before and after apamin application. Sutbraction trace of apamin sensitive tail current is shown at right panel. (**b**) Apamin sensitive currents apparently exist in CA1 pyramidal neurons and are absent in SK2 knock out mice, and increased in SK2 overexpressing mice (SK2tTA), but same in SK1 or SK3 null mice. (**c**) Average amplitude of apamin sensitive tail current (left) and slow IAHP (right).

Figure 4. Synaptic SK2 channels modulate EPSPs.



(Ngo-Anh et al., 2005; Lin et al., 2008; Adelman et al., 2012)

Figure 4. (**a**) Image of a CA1 pyramidal neuron, with a dendritic spine highlighted. Double immunogold EM for SK2 (10nm) and NR1 (20nm) in the dendritic spine of CA1 pyramidal neuron (s) shows colocalization of SK2 channel and NMDAR in the PSD. (**b**) Schematic demonstrates that presynaptic glutamate release activates AMPARs that depolarize the spine. This relieves Mg^{2+} block of

NMDARs and allows Ca^{2+} influx that activates nearby SK2-containing channels that provide an outward repolarizing K^+ conductance that decreases the EPSP and the spine Ca^{2+} transient. Upon the induction of LTP, synaptic SK2 channels undergo PKA dependent endocytosis that requires prior exocytosis of GluA1-containing AMPARs, contributing expression of LTP. (**c**) Left, average EPSP in control conditions, in the presence of apamin or in the presence of apamin plus AP5. Right, spine Ca^{2+} transient under control conditions and in the presence of apamin.

Chapter II.

Membrane palmitoylated protein 2 is a synaptic scaffold protein required for synaptic SK2-containing channel function

The contents of chapter II have been published (Kim et al., 2016).

Introduction

At most excitatory synapses in the central nervous system, such as the Schaffer collateral to CA1 synapses in the stratum radiatum of the hippocampus, excitatory neurotransmission is largely mediated by ionotropic AMPA-type (α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA-type glutamate receptors. Yet an emerging theme is that several conductances that limit membrane depolarization also make substantial contributions to the integrated excitatory post-synaptic potential (EPSP). For example, synaptically evoked Ca^{2+} influx into dendritic spines activates apamin-sensitive SK2-containing channels (small conductance Ca^{2+}-activated K^+ channels type 2; KCNN2), and their outward K^+ conductance shunts the AMPAR-mediated depolarization, effectively reducing the EPSP [1,2]. Kv4.2-containing channels are expressed in spines, close to, but not in the PSD [3]. Synaptic activity evokes Ca^{2+} influx through R-type voltage-gated Ca^{2+} channels in spines that boosts nearby Kv4.2-containing A-type K^+ channels to further decrease the AMPA-mediated depolarization [4]. In addition, Ca^{2+}-activated Cl^- channels are expressed in the spines and provide further inhibitory contributions [5]. Indeed, the sum of these repolarizing conductances may reduce the depolarizing AMPA-NMDA component by more

than 50%. It is likely that each of these components can be regulated by a variety of second messenger pathways, greatly expanding the repertoire of targets to fine-tune synaptic transmission. For example, the Ca^{2+} sensitivity of SK2 channels is regulated in an activity-dependent manner by co-assembled protein kinase CK2 and protein phosphatase 2A [6,7] that are engaged by cholinergic signaling [8]. Moreover, the various contributions to synaptic responses may be dynamic, changing in response to distinct patterns of activity. Synaptic SK2-containing channels undergo protein kinase A (PKA)-dependent endocytosis upon the induction of LTP by theta burst pairing. The endocytosis of synaptic SK2-containing channels acts together with the PKA-dependent exocytosis of additional GluA1-containing AMPARs to mediate the expression of LTP [9]. Moreover, after the initial expression of LTP and loss of the SK2-containing channel contribution, homeostatic mechanisms act to re-establish the synaptic SK balance [10]. Similarly, Kv4.2-containing channels expressed in spines undergo PKA dependent endocytosis after the induction of LTP [3,11]. Therefore, the appropriate localization, spatial distribution, and orchestrated dynamics of these protein complexes provide a powerful regulator of excitatory neurotransmission and plasticity.

One class of proteins that plays a major role in synaptic organization and dynamics are the MAGUKs [12], of which there are 10 subfamilies. These modular, usually multivalent scaffolds bind to synaptic receptors, channels, and

signaling molecules to anchor them into their proper locations within the post-synaptic membrane [13], creating a spatially and temporally restricted signaling domain [11,14,15]. Thus, within the post-synaptic density of excitatory synapses PSD-95 binds to NMDARs [16], while SAP97 binds to AMPARs [17,18], and Shank and Homer may serve as modular organizers of the lattice of synaptic MAGUKs [19,20]. However, the molecular mechanisms that engender synaptic localization and dynamics to SK2-containing channels are not well understood. There are two major isoforms of SK2 that are expressed in CA1 pyramidal neurons; SK2-L (long) has an extended intracellular N-terminal domain compared to SK2-S (short) and the two isoforms co-assemble into heteromeric channels [21]. In mice that selectively lack SK2-L expression, the SK2-S channels are expressed in the plasma membrane of dendrites and dendritic spines, yet fail to become incorporated into the post-synaptic membrane. Consequently the SK2-containing channel contributions to EPSPs and plasticity are absent, and this loss of synaptic SK2-containing channel function enhances hippocampus-dependent learning tasks [22]. To identify proteins that might serve to localize synaptic SK2-containing channels, candidate SK2 interacting proteins were identified. One of them, the MAGUK protein MPP2 (membrane palmitoylated protein 2), is localized to the PSD and is essential for synaptic SK2-containing channel function.

Results

MPP2 interacts with SK2

Proteomic analyses (mass spectrometry-based analysis) of SK2-containing channels immunoaffinity-purified from rodent whole brain membrane preparations suggested that the MAGUK protein, MPP2 might be an interaction partner. To further investigate this interaction, two newly generated antibodies targeting MPP2 were tested. Probing Western blots of proteins prepared from total mouse brain with either MPP2 antibody detected a predominant band at ~55 kDa. Similarly a single band, ~60 kDa, was detected in proteins prepared from HEK293 cells expressing FLAG-tagged MPP2, but not from mock transfected cells. For both brain and HEK293, pre-incubating the antibodies with the respective immunizing antigen abolished the bands (Fig.5 A,B).

Therefore, these two antibodies were used for affinity purifications and proteomic analyses (see Methods). The results demonstrated that both antibodies robustly purified MPP2 and co-purified SK2 as well as SK3. In addition, these experiments identified DLG1 (SAP97) and Lin-homologs 7A and 7C as proteins co-assembling with MPP2 in rodent brain (Fig. 5C; Supplementary Table 1; Supplementary Fig. 1). To be certain that the proteomics results were not due to cross-reactivity of the MPP2 antibodies with SAP97, Lin7A, or Lin7C, C8-tagged versions of each of these proteins, or C8-MPP2, were expressed in HEK293 cells. Western blotting with anti-C8 antibody verified expression of each protein (Fig. 5D). Using either of the anti-MPP2 antibodies detected only C8-MPP2, engendering confidence in the proteomics results. MPP2, a member of the p55

Stardust family of MAGUK proteins, is predicted to be a 552 amino acid protein. Like most MAGUKs MPP2 is a modular protein comprised of several distinct protein-protein interaction domains. There are two predicted L27 domains, followed by a single predicted PDZ domain, and then the SH3-HOOK-GK domains.

To test for direct interaction with SK2 channels, SK2-L and SK2-S, the SK2 isoforms that contribute to synaptic SK2-containing channels [22], and either C8-MPP2 or PSD-95 were co-expressed in HEK293 cells. Immunoprecipitations were performed using an anti-SK2 antibody raised in rabbits that is directed to the common C-terminal domain of the two SK2 isoforms, or using IgG as a control. Precipitated proteins were prepared as Western blots. Probing with anti-SK2 antibody that was raised in guinea pigs and directed against the same C-terminal sequence, demonstrated equivalent SK2 expression for input and after immunoprecipitation in each sample (Supplementary Fig. 2A). Probing with anti-C8 antibody detected a band of the appropriate apparent molecular weight for C8-MPP2 in the sample co-expressed with SK2-S plus SK2-L but not in the IgG control sample (Fig. 6A). Probing with anti-PSD-95 antibody did not detect co-immunoprecipitation with SK2 (Fig. 6B). The results demonstrated that C8-MPP2 but not PSD-95 was specifically co-precipitated with SK2. However, it is possible that SAP97 interacts with SK2-S. To examine this possibility, C8-SAP97 was co-expressed in HEK293 cells together with either myc-SK2-S or GluA1, a known

SAP97 interaction partner [18]. Immunoprecipitations were performed using anti-SK2 antibody, anti-GluA1 antibody, or IgG as a control. Western blotting with anti-C8 antibody revealed that C8-SAP97 only co-immunoprecipitated when co-expressed with GluA1 (Supplementary Fig. 2B), despite equivalent levels of input and immunoprecipitated proteins (Supplementary Fig. 2C,D). To test if MPP2 interacts with the unique N-terminal domain of SK2-L that is required for synaptic localization GST pull-down experiments were performed. While PDZ interactions are important for many MAGUK-partner protein interactions, the unique N-terminal domain of SK2-L does not contain a PDZ ligand motif and using the PDZ domain from MPP2 failed to show an interaction with the N-terminal domain of SK2-L. Therefore, the SH3-HOOK-GK domain of MPP2 was employed. This domain of MPP2 as well the SH3-HOOK-GK domains from SAP97, a MAGUK scaffold of a distinct subfamily from MPP2 [13], or the SH3-HOOK-GK domain from CaCNB4, a non-canonical MAGUK protein that is a beta subunit for voltage-gated Ca^{2+} channels [23] were prepared as GST-fusion proteins, bound to glutathione-agarose beads, and used as baits for the prey, the His-tagged N-terminal domain of SK2-L. A GST-fusion protein of the C-terminal domain of Kv1.4, and a His-fusion protein of PSD-93 (Chapsyn) were also prepared as positive interaction controls [24]. Coomassie staining and Western blotting with anti-GST antibody verified equivalent amounts of the baits, either as input or bound to beads (Supplementary Fig. 3A,B). After exposure to the baits, bound prey proteins were eluted and prepared as Western blots. Probing with an anti-

His antibody showed that the SH3-HOOK-GK domain from MPP2, but not from SAP97 or CaCNB4, specifically pulled down the N-terminal domain of SK2-L. As expected, the C-terminal domain of Kv1.4 pulled down PSD-93 (Fig. 6C). Taken together these results suggest that MPP2 interacts with the unique N-terminal domain of SK2-L that is required for synaptic localization [22].

MPP2 is a synaptic MAGUK protein

To examine the anatomical localization of MPP2, the anti-MPP2 antibodies were used for immunohistochemistry on hippocampal sections. At the light level, MPP2 was expressed throughout the hippocampus, and in area CA1, MPP2 was predominantly expressed in the dendritic arbors (Fig. 7A,B). To determine the subcellular profile of MPP2 expression, pre- and post-embedding immuno-gold electron microscopy (iEM) was performed. Using pre-embedding techniques, sections from 3 animals revealed immunoparticles for MPP2 prominently labeled dendritic spines and dendritic shafts both along the plasma membrane and at intracellular sites. Post-synaptically, most immunoparticles for MPP2 were found in spines (84%; 436 particles) versus in dendrites (16%; 82 immunoparticles), with most immunoparticles in either compartment localized to the plasma membrane (84%; 365 immunoparticles in spines and 65%; 54 immunoparticles in dendrites). Next, post-embedding techniques were applied to determine if MPP2 is expressed in the post-synaptic density. Sections from 3 animals revealed that immunoparticles for MPP2 prominently labeled the post-synaptic densities of

dendritic spines, as well as being detected in dendritic shafts (Fig. 7C,D). Immunoparticles for MPP2 were not detected in *stratum pyramidale*, and were also absent pre-synaptically. Therefore, MPP2 is a synaptic MAGUK protein.

MPP2 is required for synaptic SK2-containing channel function

To test whether MPP2 expression is important for synaptic SK2 channel function, two short hairpin RNAs (shRNAs) targeting the 3' untranslated region (3' UTR) of MPP2 mRNA were co-expressed in area CA1 by *in utero* electroporation (e14-16) of a plasmid that also directed expression of the fluorescent protein, GFP. Four to five week old mice were then used to prepare fresh hippocampal slices. Whole cell current clamp recordings were made from CA1 pyramidal neurons, either transfected, as reported by GFP expression, or non-transfected control cells. Synaptic stimulations of the Schaffer collateral axons evoked EPSPs. After establishing a stable baseline, apamin (100 nM) was applied to the slices. For control cells, apamin increased EPSPs to 144.4 ± 4.3% of baseline ($n = 15$; $P < 0.0001$), consistent with previous studies [1,9,25]. In contrast, apamin did not significantly affect EPSPs from transfected CA1 pyramidal neurons (94.6 ± 3.3%; $n = 14$) (Fig. 8A-C).

To further verify the efficacy of MPP2 knock down, pre-embedding iEM was performed on hippocampal sections from MPP2 shRNA transfected animals. To identify transfected CA1 pyramidal neurons, mouse anti-GFP antibody was

detected using HRP-linked anti-mouse secondary antibody and rabbit anti-MPP2 antibody was detected using immunogold particles coupled to anti-rabbit secondary antibody. Immunoparticles were then counted in the same number of profiles belonging both to dendritic spines and dendritic shafts on single sections. In spines on neurons expressing GFP, immunoparticles for MPP2 were reduced by 81% compared to spines on non-transfected control neurons (GFP-negative: 417 particles in 60 spines; GFP-positive: 101 particles in 60 spines; $P < 0.001$). A similar reduction (78%) was seen in dendritic shafts (GFP-negative: 155 particles in 25 dendrites; GFP-positive: 44 particles in 25 dendrites; $P < 0.001$) (Fig. 9A-D).

To demonstrate that the effects of shRNA transfection were specifically due to MPP2 knock down, the shRNA (GFP) plasmid was co-transfected with a plasmid that directed expression of shRNA-immune MPP2 and a plasmid that expressed the red fluorophore, mApple. In doubly transfected neurons, apamin increased EPSPs similar to control, non-transfected neurons ($148.2 \pm 4.2\%$; $n = 13$; $P < 0.0001$, compared to shRNA knock down) (Fig. 10A,B). Thus the consequences of the shRNA expression are mediated by knock down of MPP2.

MPP2 selectively affects synaptic SK2-containing channels

There are at least two functionally distinct populations of apamin-sensitive SK2-containing channels in CA1 pyramidal neurons. One population resides in the

spines and is activated by synaptically evoked NMDAR-dependent Ca^{2+} influx [1]. Another population resides in the dendrites, and may be activated by somatic voltage steps that induce Ca^{2+} influx through voltage gated Ca^{2+} channels [26-28]. To determine if MPP2 knock down specifically affects synaptic SK2-containing channels or also affects SK2-containing channels in the dendrites transfected or control neurons were clamped at -55 mV and stepped to 20 mV; tail currents were elicited upon stepping back to -55 mV (Fig. 10A). The apamin sensitive component of the tail current (Fig. 11A inset) effectively measures the SK2-containing channel component activated by somatic depolarization and showed that MPP2 knock down did not significantly affect somatic activation of SK2-containing channels (ctrl: 41.9 ± 8.3 pA, n = 13; transfected: 53.7 ± 10.5, n = 7; P = 0.44) (Fig. 11A,B). These results show that knocking down MPP2 expression disrupts the synaptic SK2-containing channel component but does not affect the channels in the dendrites that are activated by somatic voltage pulses.

Loss of MPP2 reduces LTP

SK channel activity modulates the induction of synaptic plasticity [29] and the endocytosis of synaptic SK2-containing channels contributes to the expression of LTP [9]. To determine the consequences of MPP2 knock down and loss of synaptic SK2-containing channel activity on LTP, a theta burst pairing protocol was delivered in which Schaffer collateral stimulations were paired with back-

propagating action potentials evoked by somatic current injections. In non-transfected CA1 pyramidal neurons, this induced LTP of 407.9 ± 46.3% (n = 8), while in transfected, MPP2 knock down neurons, LTP was significantly reduced (243.0 ± 14.8%; n = 13; $P < 0.001$) (Fig. 12). Consistent with the loss of synaptic SK2-containing channels, MPP2 knock down reduces LTP.

Discussion

The results presented here identify the synaptic MAGUK protein, MPP2 (p55) that is required for synaptic SK2-containing channel function. Synaptically evoked EPSPs in CA1 pyramidal neurons are increased by the SK channel blocker, apamin, but in CA1 pyramidal neurons expressing shRNAs directed against MPP2 apamin has no effect. MPP2 knock down selectively affects synaptic SK2-containing channel function, as the SK2-containing channels expressed in the dendrites that are activated by somatic voltage steps are not altered. Consistent with the effects of MPP2 on synaptic SK2-containing channels, MPP2 knock down reduces the expression of TBP-induced LTP by ~30%. This is slightly more than the component of LTP attributed to SK2 endocytosis in untransfected CA1 pyramidal neurons, ~17% [9]. This might reflect effects of MPP2 knock down on other interaction partners (see below).

Previous results showed that synaptic SK2-containing channels are heteromeric assemblies that contain two isoforms of SK2, SK2-S and SK2-L. Compared to SK2-S, SK2-L has an additional 207 amino acids in the intracellular N-terminal domain and SK2-S is otherwise entirely contained in SK2-L [21]. In a transgenic mouse selectively lacking SK2-L, the SK2-S channels are expressed in the dendrites and even in the plasma membrane of dendritic spines, but they are specifically excluded from the PSD, and apamin fails to boost synaptically evoked EPSPs. Re-expressing SK2-L reinstates synaptic function as measured by apamin sensitivity of EPSPs [22]. These results implicated the unique N-terminal domain of SK2-L in directing SK2-containing channel synaptic localization and function, and suggested that the N-terminal domain of SK2-L might interact with a partner protein to engender PSD localization. Indeed, MPP2 binds to the unique N-terminal domain and knocking down MPP2 expression phenocopied the effect of SK2-L deletion on synaptic responses. MAGUK proteins recruit and stabilize AMPA and NMDA receptors in the PSD. Our results suggest that MPP2 similarly serves to stabilize SK2-containing channels in the PSD.

MPP2 is a member of the p55 Stardust subfamily of MAGUK scaffold proteins, named after the founding member MPP1, the major palmitoylated protein in erythrocytes [30]. Similar to other MAGUK scaffold proteins, MPP2 is modular, consisting of two L27 domains that may mediate homo- or heterophilic interactions, a single PDZ domain followed by SH3-HOOK-GK domains.

Biochemical studies showed that MPP2 is enriched in the PSD fractions of rat brain, and pull down assays to test for interactions suggested MPP2 may interact with itself as well as a number of other synaptic proteins, among them are PSD-95, SAP97, GKAP, CASK, GRIP, neuroligin, and CaMKII. The SH3-HOOK-GK domain of MPP2 was implicated in mediating these interactions [31], similar to the interaction between MPP2 and the N-terminal domain of SK2-L.

The results presented here, using an unbiased approach also identified SK3 as immunopurifying with MPP2. SK2 and SK3 can form heteromeric channels in brain [21] and SK3 is expressed in CA1 pyramidal neurons [32]. Different from SK2 that has two N-terminal isoforms, there is only one SK3 N-terminal isoform and it is similar to the extended N-terminal domain of SK2-L, harboring several islands of homology that might mediate interactions with MPP2.

The proteomics analyses of MPP2 also identified three additional scaffold proteins, the MAGUK protein, DLG1 (SAP97) as well as Lin7A and Lin7C as MPP2 interacting proteins. In epithelial cells, MPP7, a closely related member of the p55 Stardust family, dimerizes with Lin7 proteins, an interaction mediated by the C-terminal L27 domain, and the dimeric complex then associates with DLG1 via the N-terminal L27 domain that is insufficient to mediate DLG1 interactions in the absence of bound Lin7 [33]. DLG1 also binds the C-terminal PDZ ligand on the GluA1 subunit of AMPA receptors [18]. Indeed, upon the induction of LTP at

Schaffer collateral to CA1 synapses, additional GluA1-containing AMPA receptors undergo exocytosis at a perisynaptic site followed by translocation into the post-synaptic membrane, increasing the AMPA component of EPSPs [34]. This exocytosis is dependent upon the PDZ ligand at the C-terminus of the GluA1 subunit [10,34,35], and exocytosis of GluA1-containing AMPA receptors is prerequisite to SK2-containing channel endocytosis; specifically blocking GluA1-containing AMPA receptor exocytosis prevents the rapid, subsequent endocytosis of SK2-containing channels [10]. Moreover, immunopurification of AMPA receptors from whole brain identified MPP2 as a protein that co-purified with AMPA receptors [36]. Synaptic SK2-containing channels reside in very close proximity to synaptic NMDA receptors within the PSD, providing a molecular microdomain that facilitates their functional coupling [1,9]. PSD-95 interacts with NMDARs [37], is crucial for the proper synaptic localization of ionotropic glutamate receptors [38,39], and interacts with SAP97 [40]. It will be interesting to determine whether MPP2 additionally interacts, directly or indirectly with PSD-95 to maintain the spatial synaptic relationship between SK2-containing channels and NMDA receptors. MPP2 contains multiple different protein-protein interaction domains that may bind not only receptors and channels but, additionally, signaling molecules as well as connections to the cytoskeleton. These observations raise the possibility that there is a dynamic protein lattice encompassing SK2-containing channels, AMPARs and NMDARs, and regulatory proteins that is woven together by molecular interactions between scaffold

proteins to precisely tune synaptic responses during basal neurotransmission and plasticity.

Materials and Methods

Animal Care

All procedures involving animals were performed in accordance with the guidelines of Oregon Health and Science University (Portland, OR), animal care protocol number: IP00000191; University of Freiburg (Freiburg, Germany), Regierungspra¨sidium Freiburg, AZ: 35–9185/G-12/47; and University of Castilla-La Mancha (Albacete, Spain).

Molecular biology

MPP2 shRNAs were designed using an online algorithm (http://sirna.wi.mit.edu). Two sequences targeting the 3'UTR of mouse MPP2 mRNA (NM_016695; 1811-1833; 3620-3642) were synthesized for shRNA expression and cloned into a vector that drove their expression from the U6 promoter. This same plasmid also directed GFP expression from the ubiquitin promoter. For rescuing MPP2 knock down, the MPP2 shRNA plasmids (GFP) were co-transfected with a plasmid expressing the MPP2 coding sequence, cloned between the CAGG promoter and the 3' UTR from bovine growth hormone. This plasmid was co-transfected with a plasmid directing mApple expression from the ubiquitin promoter.

Antibodies

To generate antibodies to MPP2, rabbits were immunized with synthetic peptides representing amino acids 116-145 and 480-507 of mouse MPP2 (NP_057904). To test the antibodies using Western blots, mouse whole brains were homogenized in ice-cold homogenization buffer (0.32 M sucrose, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycoltetraacetic acid, 10 mM Tris-HCl, pH 7.2, 0.4 mM phenylmethylsulfonyl fluoride). The resultant homogenate was subjected to centrifugation at 1000 g for 10 min to remove nuclei and debris. The protein concentration was determined with Lowry's method. Human embryonic kidney 293T (HEK293T) cells were transfected with pFLAG-CMV vector (Clontech, Palo Alto, CA) encoding mouse FLAG-MPP2 in a 10 cm dish. Cells were harvested in 0.5 ml of PBS. Homogenates and suspended cells were mixed with an equal volume of 2 x sodium dodecyl sulfate (SDS) sampling buffer (63 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.002% bromophenol blue), and denatured with 50 mM (±)-dithiothreitol at 55 °C for 30 min. Proteins (100 μg of brain homogenates and 0.5 μl of cell lysates) were separated using 10% SDS-polyacrylamide gel electrophoresis, and electroblotted onto an Immobilon-P Transfer Membrane (Millipore, Billerica, MA). After blocking with 5% skimmed milk for 30 min, blotted membranes were incubated with primary antibodies (1 μg/ml) for 1 h, then with peroxidase-conjugated secondary antibodies for 1 h (Jackson ImmunoResearch, West Grove, PA; 1:10000). Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.1% Tween-20 was used as the dilution and washing buffer. Immunoreactions were

visualized with the ECL chemiluminescence detection system, and captured using an ImageQuant LAS 500 (GE Healthcare, Buckinghamshire, UK). For specificity control, anti-MPP2a and anti-MPP2b (1 μg/ml) were mixed with 20 μg/ml of GST fusion proteins of the respective immunizing antigen.

Anti-SK2 antibodies have been previously characterized [9,22,41]. Mouse monoclonal anti-Myc (05-419), mouse monoclonal anti-GST (05-311), rabbit polyclonal anti-GluA1 (AB1504), and mouse monoclonal anti-GluA1 (MAB2263) antibodies were from EMD Millipore (Billerica, MA). Anti-6XHis mouse monoclonal antibody (37-2900) and anti-PSD-95 mouse monoclonal antibody (MA1-045) were from Thermo Scientific (Waltham, MA). Anti-GFP (ab92) mouse monoclonal antibody was from Abcam (Cambridge, MA). Mouse monoclonal C8 antibody was obtained as supernatant from Chessie 8 cells, LN 10300SP (National Cell Culture Center, Biovest, Minneapolis, MN). Secondary antibodies were from Santa Cruz Biotechnology (Dallas, TX), unless otherwise noted.

Proteomic analysis

Proteomic analysis of MPP2 from rat brain was performed as described previously [36,42,43]. Briefly, affinity-purifications (APs) with anti-MPP2a, anti-MPP2b and pre-immunization immunoglobulins (IgG) were performed on rat brain membrane fractions solubilized with CL-91 (Logopharm GmbH, March, Germany) and whole eluates were subjected to high-resolution mass

spectrometry. Proteins retained in APs and identified by mass spectrometry were quantified by integration of peptide m/z signal intensities over time (peak volumes, PVs) that were extracted from FT full scans using MaxQuant (v.1.4.1.2; http://www.maxquant.org; with integrated off-line mass calibration). Relative abundance of proteins in anti-MPP2 samples versus control (abundance ratio or rPV) was determined by the TopCorr method [43] as the median of either (i) six individual peptide PV ratios of the best correlating protein-specific peptides (as determined by Pearson correlation of their abundance values; TopCorr6) or (ii) the 50% best of all individual peptide PV ratios (Top50). The linear dynamic range of the TopCorr method is about 4 orders of magnitude (detailed in [43]). The data used for rPV determination (including peptide sequences, PV and rPV values, as well as the respective medians and selections methods) are summarized in Supplementary Table 1. The coverage of the primary sequences of all proteins shown in Figure 1C is presented in Supplementary Figure 1.

Co-immunoprecipitation

HEK293 cells were transfected using Lipofectamine 2000 (Thermo Scientific, Waltham, MA). After 48 hours cells were washed, collected with PBS buffer, and pelleted. Cells were then solubilized with lysis buffer (20mM HEPES, pH 7.5, 150mM NaCl, 10% glycerol, 2mM EDTA, 1mM PMSF, and protease inhibitors (Hoffman La Roche, Basel, Switzerland) containing 1% β-D-dodecyl maltoside (Sigma-Aldrich, St. Louis, MO) for 30 min at RT. The lysate was centrifuged at

14,000 rpm for 20 min at 4°C. The supernatant was incubated with the indicated primary antibody for 30 min then 20 μl of protein A/G agarose beads (Thermo Scientific, Waltham, MA) were added for incubation overnight at 4°C with rotation. The beads were washed 3 times in washing buffer (mM) (20 HEPES, pH 7.5, 150 NaCl, 10 KCl, 2 EDTA, 10% glycerol). Proteins were eluted with 40 μl of 2X SDS sample buffer at 37°C. Bound and eluted proteins were subsequently separated by SDS-PAGE and transferred to PVDF membrane (BioRad, Hercules, CA). After blocking with 5% skim milk for 1 hour, the membrane was probed with the indicated antibodies over night at 4°C. HRP-conjugated secondary antibodies were applied for 30 min at RT. Blots were detected with SuperSignal ECL (Thermo Scientific, Waltham, MA) and developed with GeneMate Blue film (BioExpress, Kaysville, UT).

GST pull-downs

The N-terminal domain of SK2-L (NP_001299834; 67-273) was cloned into pET33b for His tagged expression. Plasmids for expression of His-tagged rat PSD-93 (P85-T280) and GST-Kv1.4-ct (H570-V665) were gifts from Dr. Paul Slesinger [24]. For GST-fusion proteins, the SH3-HOOK-GK domains of mouse MPP2 (216-552; NM_016695.3), SAP97 (577-927; NM_007862.2), and CaCNB4 (45-371; NM146123.2) were cloned into pGex4T3. Expression levels of the GST-baits were determined by Coomassie staining and Western blotting with anti-GST antibody (Supplemental Fig. 3), or for His-prey, with anti-His antibody (Fig. 2).

GST pull-downs were performed as previously described [7], with minor modifications. Glutathione agarose beads (Sigma-Aldrich, St. Louis, MO) were resuspended and 50 µl of slurry was used per reaction. Beads were washed one time with pull-down wash buffer (PWB; 20 mM HEPES, pH 7.8, 10% glycerol, 100 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT), 0.1% Igepal CA-630 (Sigma-Aldrich, St Louis, MO)) and then incubated at 4°C for 2 h with bacterial lysate containing the GST-fusion protein. Bead–protein complexes were washed one time with PWB followed by a 5 min wash at 4°C with PWB plus 0.1% BSA, and two 1 min washes with PWB. These "baits" were incubated at 4°C overnight with bacterial cell lysate from cultures expressing the His-tagged "prey", washed 4 times with PWB, added to SDS sample buffer, heated at 95°C for 5 minutes and resolved by SDS-PAGE. Following transfer the Western blot was probed with anti-His antibody.

Immunoelectron microscopy

Immunohistochemical reactions at the electron microscopic level were carried out using immunogold methods as described previously [44].

Pre-embedding immunohistochemistry. Single and double labelling methods were used. Briefly, in single labelling experiments, free-floating sections were incubated in 10% NGS diluted in TBS. Sections were then incubated in anti-MPP2 antibodies (1–2 µg/ml diluted in TBS containing 1% NGS), followed by incubation in goat anti-rabbit IgG coupled to 1.4 nm gold (Nanoprobes Inc.,

Yaphank, NY). Sections were postfixed in 1% glutaraldehyde and washed in double-distilled water, followed by silver enhancement of the gold particles with an HQ Silver kit (Nanoprobes Inc., Yaphank, NY). In co-labelling experiments, GFP immunoreactivity was visualized by the immunoperoxidase reaction, and MPP2 immunoreactivity was revealed with the silver-intensified immunogold reaction. Sections were then treated with osmium tetraoxide (1% in 0.1 M PB), block-stained with uranyl acetate, dehydrated in graded series of ethanol and flat-embedded on glass slides in Durcupan (Sigma-Aldrich, St. Louis, MO) resin. Regions of interest were cut at 70–90 nm on an ultramicrotome (Reichert Ultracut E, Leica Biosystems, Barcelona, Spain) and collected on 200-mesh copper grids. Staining was performed on drops of 1% aqueous uranyl acetate followed by Reynolds's lead citrate.

Post-embedding immunohistochemistry. Briefly, ultrathin 80-nm-thick sections from Lowicryl-embedded blocks of the hippocampus were picked up on coated nickel grids and incubated on drops of a blocking solution consisting of 2% human serum albumin in 0.05 M TBS and 0.03% Triton X-100. The grids were incubated with MPP2 antibodies (10 µg/ml in 0.05 M TBS and 0.03% Triton X-100 with 2% human serum albumin) at 28 °C overnight. The grids were incubated on drops of goat anti-rabbit IgG conjugated to 10 nm colloidal gold particles (Nanoprobes Inc., Yaphank, NY) in 2% human serum albumin and 0.5% polyethylene glycol in 0.05 M TBS and 0.03% Triton X-100. The grids were then

washed in TBS and counterstained for electron microscopy with saturated aqueous uranyl acetate followed by lead citrate.

Ultrastructural analyses were performed in a Jeol-1010 electron microscope (Synaptic Structure Laboratory, School of Medicine, University of Castilla-La Mancha, Albacete, Spain). Electron photomicrographs were captured with an ORIUS SC600B CCD camera (Gatan, Munich, Germany). Digitized electron images were modified for color, brightness, and contrast with Adobe Photoshop, version 7.0. Labelled structures were classified based on unambiguous morphological information in each section. Axon terminals were identified by the presence of synapses and small round and/or large granular vesicles. Synapses were identified as parallel membranes separated by widened clefts that were associated with membrane specializations. Synapses displaying a prominent density on the post-synaptic side were characterized as asymmetrical. Dendritic spines were identified as small protrusions exhibiting membrane continuity with the dendritic shaft. Significance of immunogold particle labelling in GFP-positive versus GFP-negative spines and dendrites was assessed by ANOVA.

In utero electroporation

Timed-pregnant mice were anesthetized with isofluorane, their abdominal cavity cut open, and the uterine horns/sac exposed. Approximately 2 µl of DNA solution (~2 mg/ml) was injected into the lateral ventricle of e14-e16 embryos, using a glass pipet pulled from thin walled capillary glass (TW150F-4, World Precision

Instruments, Sarasota, FL) and a Picospritzer III microinjection system (Parker Hannifin, Hollix, NH). The head of each embryo within its uterine sac was positioned between tweezer-type electrodes (CUY650P10; Sonidel Ltd., Dublin, Ireland), and 5 square electric pulses (35V; 50 ms; 1 s intervals) were passed using an electroporator (CUY21; Sonidel Ltd., Dublin, Ireland). After electroporation, the wall and skin of the abdominal cavity of the pregnant mouse was sutured-closed, and embryos were allowed to develop normally.

Slice preparation

All procedures were performed in accordance with the guidelines of Oregon Health and Science University (Portland, OR) and University of Castilla-La Mancha (Albacete, Spain). Hippocampal slices were prepared from 4-5-week-old C57BL/6J mice. Animals were anesthetized with isoflurane and decapitated. The cerebral hemispheres were quickly removed and placed into cold artificial CSF (ACSF) equilibrated with carbogen (95% O_2/5% CO_2). Hippocampi and cortex were removed, placed onto an agar block, and transferred into a slicing chamber. Transverse hippocampal slices (300–350 µm) were cut with Leica VT1200s (Leica Biosystems, Buffalo Grove, IL) and transferred into a holding chamber containing regular ACSF. Slices were incubated at 34°C for 30 min and then at room temperature for ≥1 h before recordings were performed. The slicing solution consisted of sucrose-ACSF (in mM): 70 sucrose, 80 NaCl, 2.5 KCl, 21.4 $NaHCO_3$, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgCl_2$, 1.3 ascorbic acid, 20 glucose and

regular ACSF consisted of (in mM): 125 NaCl, 2.5 KCl, 21.5 $NaHCO_3$, 1.25 NaH_2PO_4, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 15 glucose, equilibrated with carbogen.

Electrophysiology

CA1 pyramidal cells were visualized with IR/DIC optics (Olympus BX51W1; Olympus Scientific Solutions, Waltham, MA) and a CCD camera. Whole-cell, patch-clamp recordings were obtained from CA1 pyramidal cells using a Multiclamp 700B (Molecular Devices, Sunnyvale, CA), digitized using an ITC-18 analog-to-digital converter, and transferred to a computer using Patchmaster software (Heka Instruments Incorporated, Bellmore, NY). Patch pipettes (open pipette resistance, 2-4 MΩ) were filled with (in mM) 133 K-gluconate, 4 KCl, 4 NaCl, 1 MgCl2, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4 MgATP, 0.3 Na_3 guanosine triphosphate (Na_3GTP), and 10 K_2-phosphocreatine (pH 7.3). Electrophysiological records were filtered at 3.3 kHz and sampled at 10 kHz. Series resistance was electronically compensated to greater than 70%. A bias current was applied to maintain the membrane potential in current clamp at -65 mV. The input and residual series resistance in current clamp was determined from a 20 pA hyperpolarizing pulse applied at the end of each sweep. The input resistance in voltage clamp was determined from a 5 mV hyperpolarizing pulse applied at the beginning of each sweep. All recordings were from cells with a resting membrane potential between -75 and -60 mV and a stable input resistance. All electrophysiological recordings were performed at 22-24˚C and

data were not corrected for a junction potential of ~15 mV of the internal solution with respect to the bath ACSF.

Synaptic stimulation

EPSPs were recorded in whole-cell mode. Capillary glass pipettes (tip diameter, ~5 μm) filled with ACSF and connected to Digitimer constant current stimulus isolation unit (AutoMate Scientific, Berkeley, CA) were used to stimulate pre-synaptic axons in stratum radiatum as described in Results. SR95531 (2-10 μM) and CGP55845 (1 μM) were present to reduce $GABA_A$ and $GABA_B$ contributions, respectively. To prevent epileptic discharges in the presence of GABAergic blockers, the CA3 region was microdissected out of slices used for EPSP recordings. LTP was induced by a theta burst pairing protocol, as previously described [9].

Data analysis

Data were analyzed using IGOR (WaveMetrics, Lake Oswego, OR). Data are expressed as mean ± SEM. Paired two sample *t* tests were used to determine significance of data in the same pathway, and Non-parametric Wilcoxon Mann-Whitney two-sample rank test was used to determine significance between groups of data; $p < 0.05$ was considered significant.

Chemicals and solutions

D-AP5, CNQX, CGP55845, and SR95531 were obtained from Tocris Bioscience (Ellisville, MO). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless specified. All perfusing solutions were modified from regular ACSF unless otherwise noted.

Acknowledgements

We thank Dr. Paul Slesinger for Kv1.4 and PSD-93. We also thank Ms. Lori Vaskalis for expert graphics design. This work was supported by grants from the NIH to JM and JPA (MH093599 and NS038880) and to RL from the Spanish Ministry of Education and Science (BFU-2012-38348), European Union (HBP - Project Ref. 604102) and Junta de Comunidades de Castilla-La Mancha (PPII-2014-005-P).

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Figure 5. MPP2 interacts with SK2.

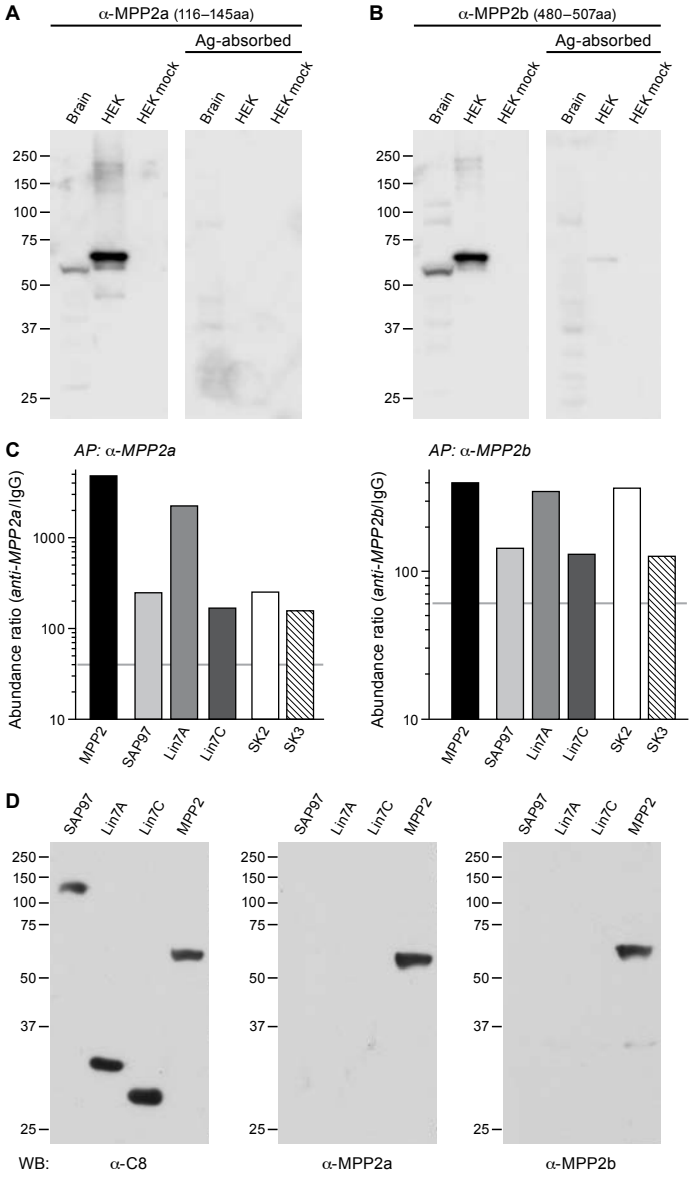


Figure 5. A, B) Western blots were prepared using mouse brain homogenate (brain; 100 μg) or HEK293 cell extracts (1% of 10 cm plate), either transfected to express FLAG-MPP2 (HEK) or empty plasmid (HEK mock). Duplicate lanes were prepared and one set was probed with the indicated MPP2 antibody (left panels) while the second set was probed with the same antibody after pre-absorbing with

the immunizing antigen (right panels). Native MPP2 and transfected FLAG-MPP2 were detected only in the left panels. C) Bar graphs illustrating abundance ratios determined for the indicated proteins in affinity-purifications with anti-MPP2 antibodies and IgG as a negative control. Horizontal lines denote threshold for specificity of co-purification. D) Western blots of proteins prepared from HEK293 cells transfected with C8-tagged SAP97, Lin7A, Lin7C, and MPP2, and probed using anti-C8 antibody (left), anti-MPP2a antibody (middle) and anti-MPP2b antibody (right). The MPP2 antibodies recognized only MPP2.

Figure 6. MPP2 interacts with the N-terminal domain of SK2-L.

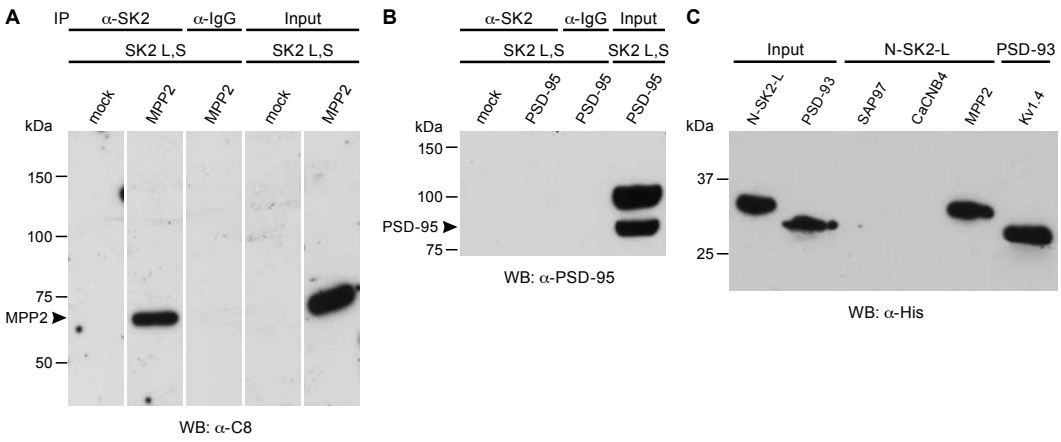


Figure 6. A) Co-immunoprecipitations. Western blots prepared from HEK cell lysates expressing SK2-S and SK2-L, either alone (mock) or together with C8-MPP2, immunoprecipitated with either anti-SK2 antibody or IgG (control), and probed for C8-MPP2. Adjacent blot shows input of MPP2. B) A similar experiment except using PSD-95 instead of C8-MPP2. PSD-95 is expressed but does not co-IP with SK2. C) GST-pull-downs. Blot probed with anti-His antibody shows input prey proteins, His-SK2-L N-terminal domain and His-PSD-93. After exposure to GST-baits representing SH3-HOOK-GK domains of SAP97, CaCNB4, or MPP2, the His-SK2-L N-terminal domain was specifically retained by GST-MPP2. Positive control shows interaction between GST-C-terminal domain of Kv1.4 and His-PSD-93. Co-immunoprecipitations and GST-pull-downs were performed in triplicate.

Figure 7. Localization of MPP2 in the hippocampus.

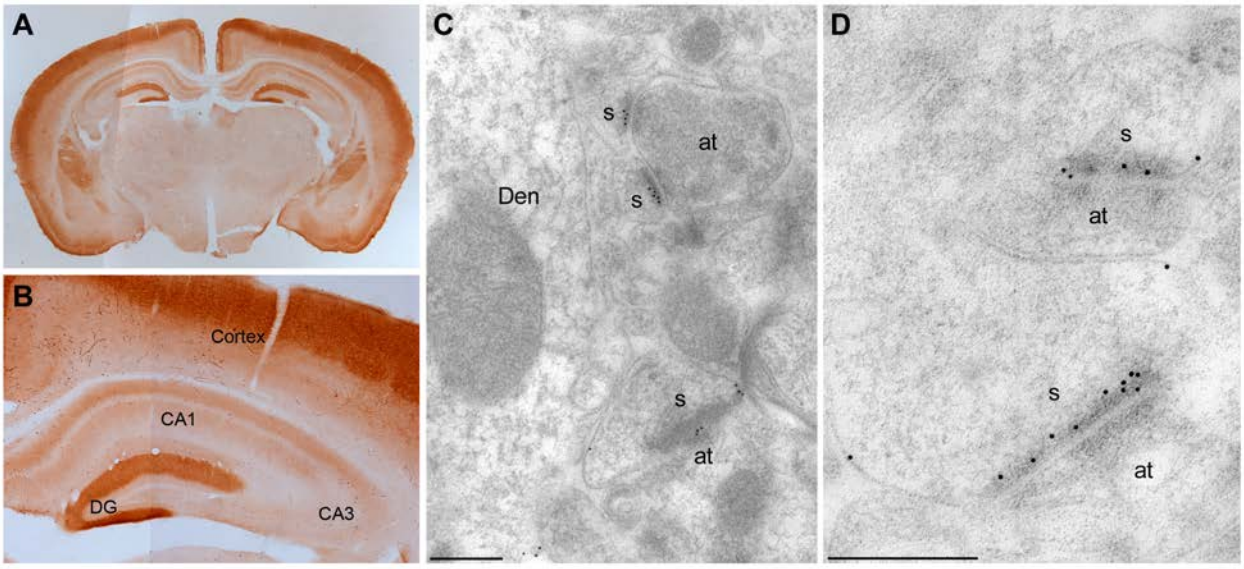


Figure 7. A,B) Light microscopic images of anti-MPP2 antibody labelling in hippocampus. MPP2 was present throughout the hippocampus and was prominent in the dendritic arbors of area CA1 C,D) Electron micrographs of the hippocampus showing immunoparticles for MPP2 in the *stratum radiatum* of the CA1 region of the hippocampus, as detected using a post-embedding immunogold method. Immunoparticles for MPP2 were detected along the PSD of dendritic spines (s) of CA1 pyramidal cells establishing asymmetrical synapses with axon terminal (at), as well as at extrasynaptic sites of dendritic spines (s). Den, dendritic shaft. Scale bars: C,D: 0.2 mm.

Figure 8. MPP2 is required for synaptic SK2-containing channel function.

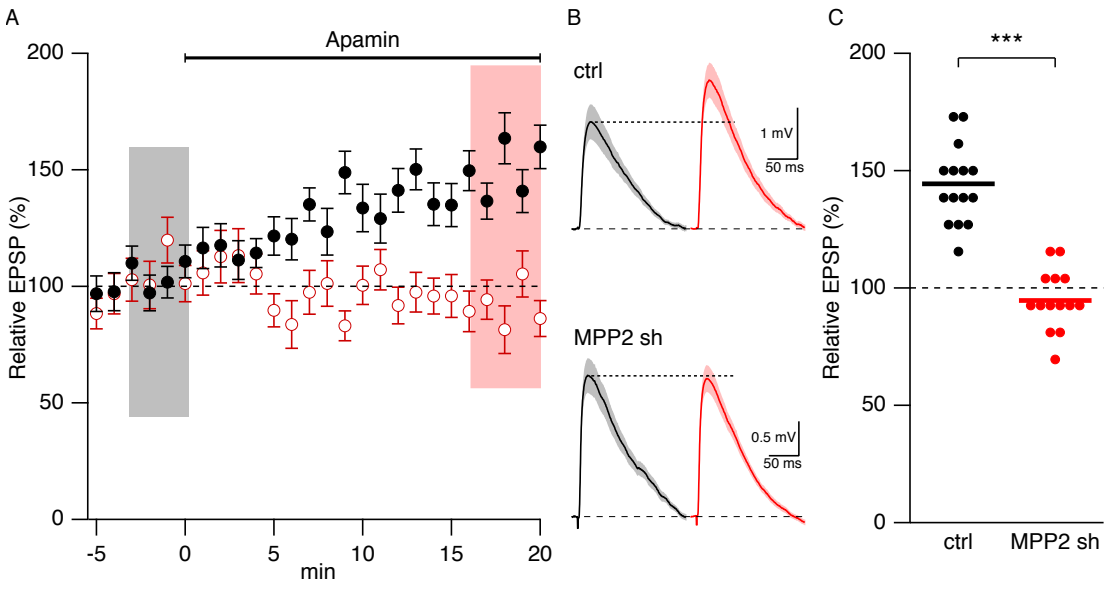


Figure 8. (A) Time course of the normalized EPSP amplitude (mean ± s.e.m.) for baseline in control ACSF (Ctrl) and during wash-in of apamin (100 nM) as indicated above in MPP2 sh transfected cells (open red symbols, n = 14) and non-fluorescent control cells (black symbols, n = 15) mice. (B) Average of 15 EPSPs taken from indicated shaded time points in aCSF (black) and 16-20 min after application of apamin (red); shaded areas are mean ± s.e.m for control non-fluorescent cells (ctrl, upper traces) and MPP2 sh transfected cells (MPP2 sh, bottom traces) (C) Scatter plot of relative EPSP peak compared to baseline from the individual slices in panel A non-fluorescent control (ctrl, black symbols) and for MPP2 sh transfected (red symbols). Horizontal bar reflects mean response.

Figure 9. Efficient knock down of MPP2 expression in CA1 pyramidal neurons.

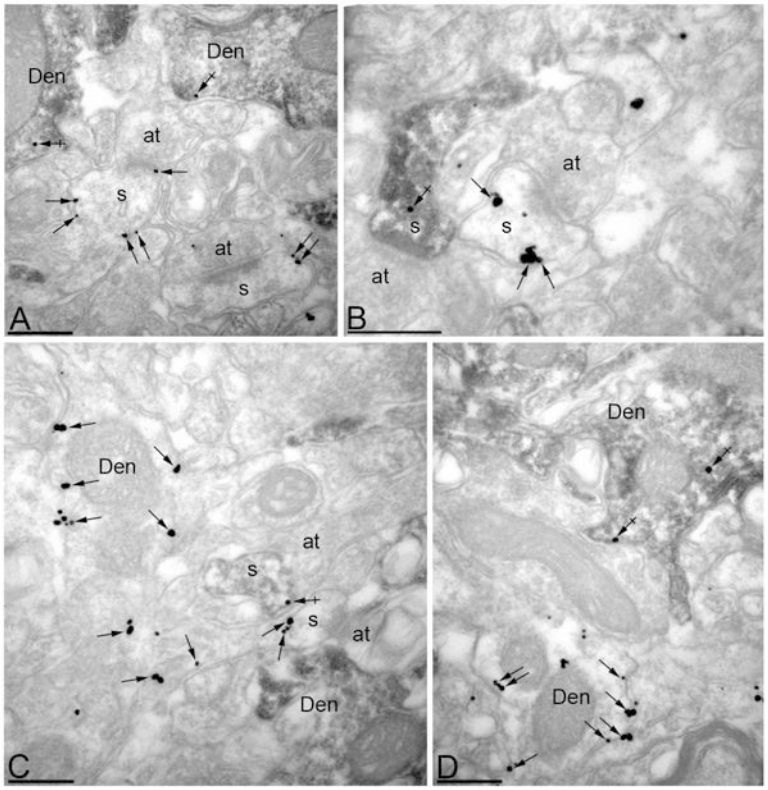


Figure 9. Immunoreactivity for MPP2 in the CA1 region of the hippocampus, as revealed using a double-labelling pre-embedding method. (A-D) The peroxidase reaction end product (HRP) indicating GFP immunoreactivity filled CA1 pyramidal cells, whereas immunoparticles for MPP2 were mainly located along the plasma membrane and at intracellular sites of pyramidal cells. Immunoparticles for MPP2 were distributed in both GFP-positive (crossed arrows) and GFP-negative (arrows) dendritic spines (s) and dendritic shafts (Den) of pyramidal cells. However, there was a striking reduction of immunoparticles for MPP2 in GFP-positive profiles compared to GFP-negative profiles (see text). at, axon terminal. Scale bars: A-D, 0.2 μm.

Figure 10. Coexpression of sh-immune MPP2 with MPP2 sh rescues synaptic SK2 function.

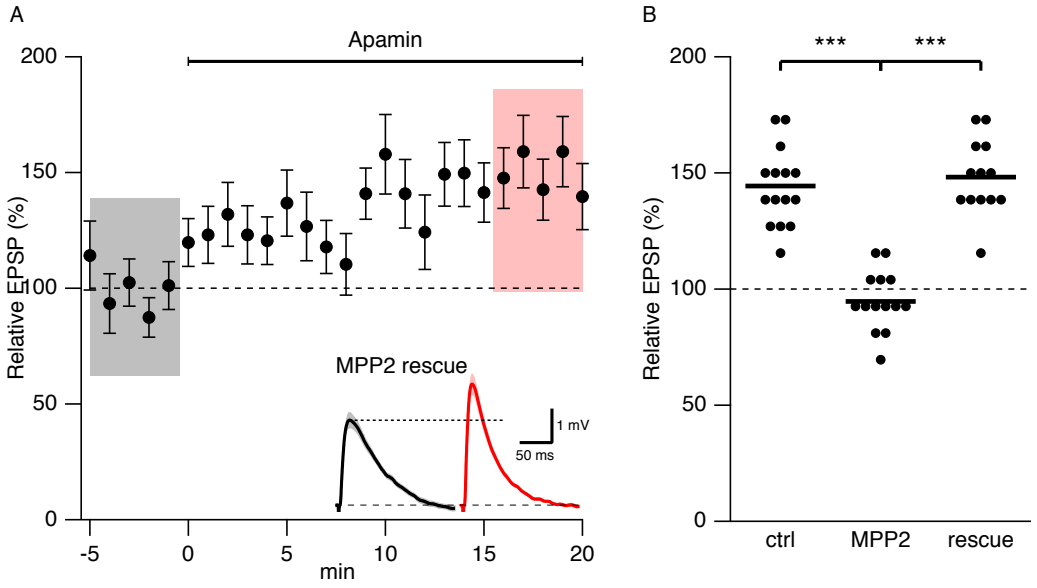


Figure 10. (A) Time course of the normalized EPSP amplitude (mean ± s.e.m.) for baseline in control ACSF and during wash-in of apamin (100 nM) as indicated above in cells transfected MPP2 sh and MPP2 sh immune (n = 13). Inset: representative cell showing average of 15 EPSPs taken from indicated shaded time points in ACSF (black) and 16-20 min after application of apamin (red); shaded areas are mean ± s.e.m. (B). Scatter plot of relative EPSP peak compared to baseline from the individual slices for non-fluorescent control, MPP2 sh transfected cells and MPP2 sh transfected with immune MPP2 (rescue). Horizontal bar reflects mean.

Figure 11. Dendritic SK channel function is not reduced by MPP2 knock down.

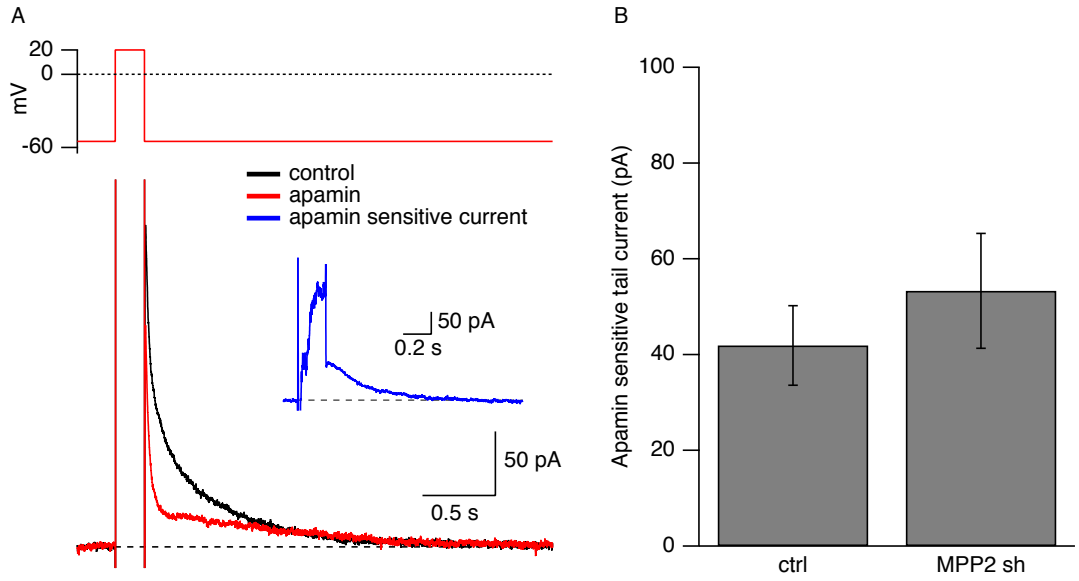


Figure 11. (A) Representative traces of voltage-clamp recordings of IAHP after a 200 msec depolarizing pulse to 20 mV in an MPP2 sh transfected cell. Apamin (red trace) blocks a component of the IAHP. Inset: subtraction of the traces before and after apamin application yielded the apamin-sensitive ImAHP. (B) Bar graph of apamin sensitive tail current measured at 100 ms following repolarization to -55 mV. Data presented as mean ± s.e.m. for control non-fluorescent cells (n = 13) and MPP2 sh transfected cells (n = 7).

Figure 12. Loss of MPP2 reduces LTP.

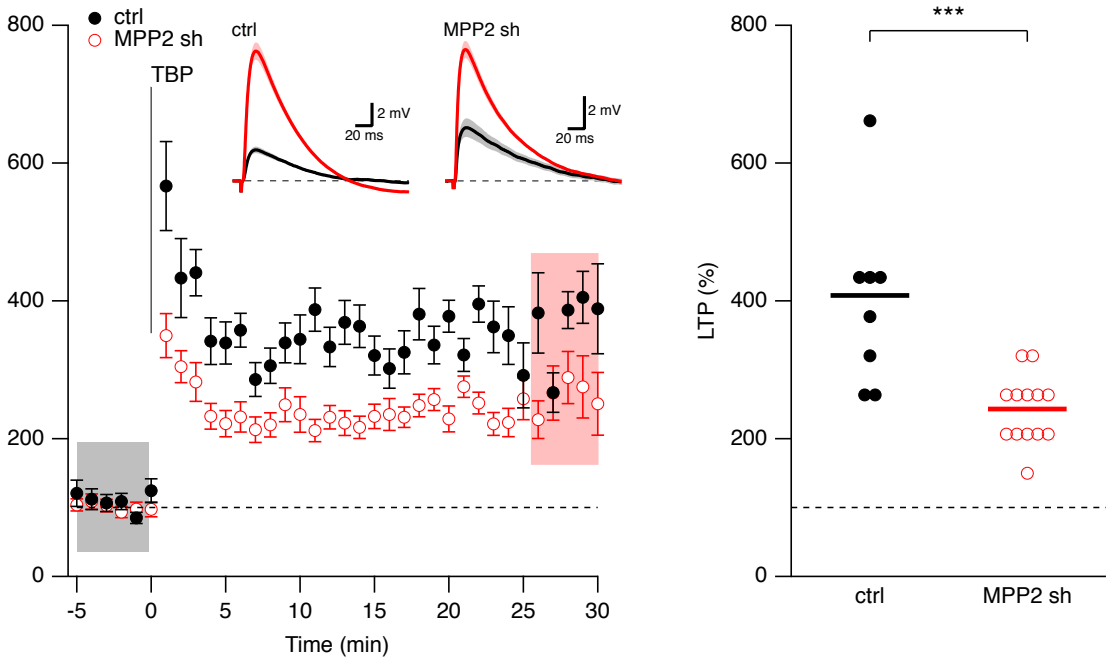


Figure 12. (A) Time course of the normalized EPSP amplitude (mean ± s.e.m.) from control non-fluorescent cells (ctrl, closed black symbols, n = 8) and MPP2 sh transfected cells (MPP2, open red symbols, n = 13). The TBP protocol was delivered at time 0. Inset: representative cell showing average of 15 EPSPs taken from indicated shaded time points in ACSF (black) and 25-30 min after the induction of LTP (red); shaded areas are mean ± s.e.m. (B). Scatter plot of relative EPSP peak compared to baseline from the individual slices for non-fluorescent control (ctrl) and MPP2 sh transfected cells. Horizontal bar reflects mean response.

Supplementary Figure 1. Coverage of the primary sequences of all proteins shown in Fig.4C.

Sequence coverage of the proteins identified in anti-MPP2 APs

MPP2_MOUSE (Q9WV34) – MAGUK p55 subfamily member 2

Coverage is **70.5%** absolute, **84.6%** relative.

MPVAATNSES AMQQVLDNLG SLPNATGAAE LDLIFLRGIM
ESPIVRSLAK
AHERLEETKL EAVRDNNLEL VQEILRDLAE LAEQSSTAAE
LARILQEPHF
QSLLETHDSV ASKTYETPPP SPGLDPTFSN QPVPPDAVRM
VGIRKTAGEH
0001 LGVTFRVEGG ELVIARILHG GMVAQQGLLH VGDIIKEVNG
0051 QPVGSDPRAL
0101 QELLRSASGS VILKILPSYQ EPHLPRQVFV KCHFDYDPAR
0151 DSLSPCKEAG
0201 LRFNAGDLLQ IVNQDDANWW QACHVEGGSA GLIPSQLLEE
0251 KRKAFVKRDL
0301 ELTPTSGTLC GSLSGKKKKR MMYLTTKNAE FDRHELLIYE
0351 EVARMPPFRR
0401 KTLVLIGAQG VGRRSLKNKL ILWDPDRYGT TVPYTSRRPK
0451 DSEREGQGYS
0501 FVSRGEMEAD IRAGRYLEHG EYEGNLYGTR IDSIRGVVAS
0551 GKVCVLDVNP
QAVKVLRTAE FVPYVVFIEA PDYETLRAMN RAALESGVST
KQLTEADLRR
TVEESSRIQR GYGHYFDLSL VNSNLERTFR ELQTAMEKLR
TEPQWVPVSW
VY

KCNN2_RAT (P70604) - Small conductance calcium-activated potassium channel protein 2

Coverage is **11.6%** absolute, **19.3%** relative.

0001 MSSCRYNGGV MRPLSNLSSS RRNLHEMDSE AQPLQPPASV
0051 VGGGGGASSP
0101 SAAAAASSSA PEIVVSKPEH NNSNNLALYG TGGGGSTGGG
0151 GGGGGGGGGS

0201 GHGSSSGTKS SKKKNQNIGY KLGHRRALFE KRKRLSDYAL
0251 IFGMFGIVVM
0301 VIETELSWGA YDKASLYSLA LKCLISLSTI ILLGLIIVYH AREIQLFMVD
0351 NGADDWRIAM TYERIFFICL EILVCAIHPI PGNYTFTWTA RLAFSYAPST
0401 TTADVDIILS IPMFLRLYLI ARVMLLHSKL FTDASSRSIG ALNKINFNTR
0451 FVMKTLMTIC PGTVLLVFSI SLWIIAAWTV RACERYHDQQ
0501 DVTSNFLGAM
0551 WLISITFLSI GYGDMVPNTY CGKGVCLLTG IMGAGCTALV
VAVVARKLEL
TKAEKHVHNF MMDTQLTKRV KNAAANVLRE TWLIYKNTKL
VKKIDHAKVR
KHQRKFLQAI HQLRSVKMEQ RKLNDQANTL VDLAKTQNIM
YDMISDLNER
SEDFEKRIVT LETKLETLIG SIHALPGLIS QTIRQQQRDF IETQMENYDK
HVTYNAERSR SSSRRRRSSS TAPPTSSESS

KCNN3_RAT (P70605) - Small conductance calcium-activated potassium channel protein 3

Coverage is **14.1%** absolute, **23.6%** relative.

MDTSGHFHDS GVGDLDEDPK CPCPSSGDEQ QQQQQPPPPS
APPAVPQQPP
GPLLQPQPPQ LQQQQQQQQQ QQQQQQQQQQ APLHPLPQLA
QLQSQLVHPG
0001 LLHSSPTAFR APNSANSTAI LHPSSRQGSQ LNLNDHLLGH
0051 SPSSTATSGP
0101 GGGSRHRQAS PLVHRRDSNP FTEIAMSSCK YSGGVMKPLS
0151 RLSASRRNLI
0201 EAEPEGQPLQ LFSPSNPPEI IISSREDNHA HQTLLHHPNA
0251 THNHQHAGTT
0301 AGSTTFPKAN KRKNQNIGYK LGHRRALFEK RKRLSDYALI
0351 FGMFGIVVMV
0401 IETELSWGLY SKDSMFSLAL KCLISLSTII LLGLIIAYHT REVQLFVIDN
0451 GADDWRIAMT YERILYISLE MLVCAIHPIP GEYKFFWTAR
0501 LAFSYTPSRA
0551 EADVDIILSI PMFLRLYLIA RVMLLHSKLF TDASSRSIGA LNKINFNTRF
0601 VMKTLMTICP GTVLLVFSIS LWIIAAWTVR VCERYHDQQD
0651 VTSNFLGAMW
0701 LISITFLSIG YGDMVPHTYC GKGVCLLTGI MGAGCTALVV
AVVARKLELT
KAEKHVHNFM MDTQLTKRIK NAAANVLRET WLIYKHTKLL
KKIDHAKVRK
HQRKFLQAIH QLRGVKMEQR KLSDQANTLV DLSKMQNVMY

DLITELNDRS
EDLEKQIGSL ESKLEHLTAS FNSLPLLIAD TLRQQQQQLL
TAFVEARGIS
VAVGTSHAPP SDSPIGISST SFPTPYTSSS SC

LIN7C_RAT (Q792I0) - Protein lin-7 homolog C

Coverage is **70.1%** absolute, **74.6%** relative.

MAALGEPVRL ERDICRAIEL LEKLQRSGEV PPQKLQALQR
VLQSEFCNAV
0001 REVYEHVYET VDISSSPEVR ANATAKATVA AFAASEGHSH
0051 PRVVELPKTE
0101 EGLGFNIMGG KEQNSPIYIS RIIPGGIADR HGGLKRGDQL
0151 LSVNGVSVEG
EHHEKAVELL KAAQGKVKLV VRYTPKVLEE MESRFEKMRS
AKRRQQT

LIN7A_RAT (Q9Z250) - Protein lin-7 homolog A

Coverage is **66.8%** absolute, **68.9%** relative.

MLKPSVTSAP TADMATLTVV QPLTLDRDVA RAIELLEKLQ
ESGEVPVHKL
0001 QSLKKVLQSE FCTAIREVYQ YMHETITVNG CPEFRARATA
0051 KATVAAFAAS
0101 EGHSHPRVVE LPKTDEGLGF NVMGGKEQNS PIYISRIIPG
0151 GVAERHGGLK
0201 RGDQLLSVNG VSVEGEHHEK AVELLKAAKD SVKLVVRYTP
KVLEEMEARF
EKLRTARRRQ QQQLLIQQQQ QQQQQPQQNH MS

DLG1_RAT (Q62696) - Disks large homolog 1

Coverage is **55.2%** absolute, **85.3%** relative.

0001 MPVRKQDTQR ALHLLEEYRS KLSQTEDRQL RSSIERVISI
0051 FQSNLFQALI
0101 DIQEFYEVTL LDNPKCVDHS KQCEPVQPGN PWESGSLSSA
0151 AVTSESLPGG
0201 LSPPVEKYRY QDEEVLPSER ISPQVPNEVL GPELVHVSEK
0251 SLSEIENVHG
0301 FVSHSHISPI KPTEAVPPSS PIVPVTPALP VPAESPVVLP STPQANPPPV
0351 LVNTDSLETP TYVNGTDADY EYEEITLERG NSGLGFSIAG

0401 GTDNPHIGDD
0451 SSIFITKIIT GGAAAQDGRL RVNDCILRVN EADVRDVTHS
0501 KAVEALKEAG
0551 SIVRLYVKRR KAFRKNHEIK LIKGPKGLGF SIAGGVGNQH
0601 IPGDNSIYVT
0651 KIIEGGAAHK DGKLQIGDKL LAVNSVCLEE VTHEEAVTAL
0701 KNTSDFVYLK
0751 AAKPTSMYIN DGYAPPDITN SSSQSVDNHV SPSSYLGQTP
0801 ASPARYSPIS
0851 KAVLGDDEIT REPRKVVLHR GSTGLGFNIV GGEDGEGIFI
0901 SFILAGGPAD
LSGELRKGDR IISVNSVDLR AASHEQAAAA LKNAGQAVTI
VAQYRPEEYS
RFEAKIHDLR ETMMNSSVSS GSGSLRTSQK RSLYVRALFD
YDKTKDSGLP
SQGLNFKFGD ILHVINASDD EWWQARQVTP DGESDEVGVI
PSKRRVEKKE
RARLKTVKFN SKTRGDKGEI PDDMGSKGLK HVTSNASDSE
SSYHEYGCSK
GGQEEYVLSY EPVNQQEVNY TRPVIILGPM KDRVNDDLIS
EFPDKFGSCV
PHTTRPKRDY EVDGRDYHFV TSREQMEKDI QEHKFIEAGQ
YNNHLYGTSV
QSVRAVAEKG KHCILDVSGN AIKRLQIAQL YPISIFIKPK
SMENIMEMNK
RLTDEQARKT FERAVRLEQE FTEHFTAIVQ GDTLEDIYNQ
VKQIIEEQSG
PYIWVPAKEK L

For peptide identification:

Database: UniProt release 2015_04 and 2015_05

Max. missed cleavages: 1

Min. peptide occurence: 2

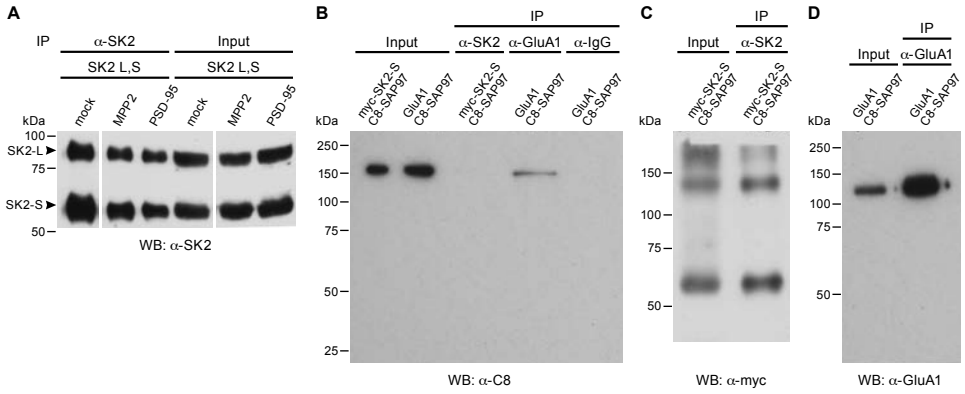
Lower mass limit: 738

Upper mass limit: 3000

Supplementary Figure 1. Peptides identified by mass spectrometry are in red; those theoretically accessible but not detected in MS-analyses are in black, and peptides not accessible to MS-analyses under the settings used (peptides with mass values below 738 Da (absolute lower mass cutoff) or above 3000 Da

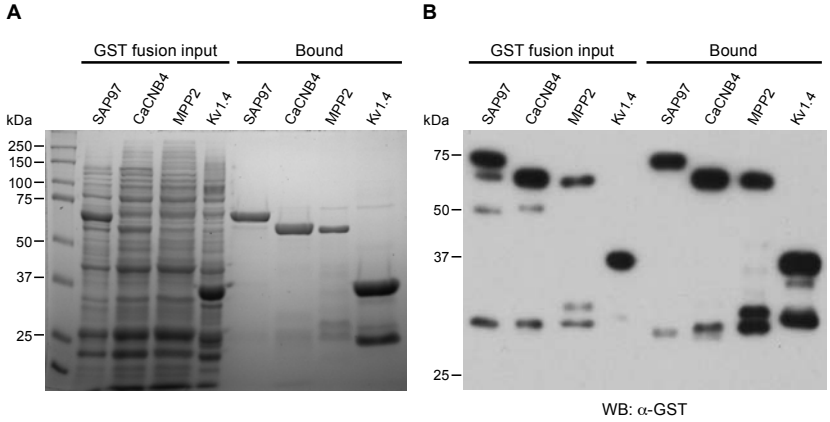
(practical mass limit of the C18 RP-HPLC separation given in grey). The sum of amino acids in red is either related to the accessible primary sequence (relative coverage) or to the entire primary sequence (absolute coverage); transmembrane domains are underlined.

Supplementary Figure 2. Co-immunoprecipitations.



Supplementary Figure 2. A) Immunoprecipitation and input of SK2-S plus SK2-L. Western blot probed with anti-SK2 antibody raised in rabbit. First three lanes show immunoprecipitates from transfected HEK293 cell lysates co-transfected with empty vector (mock), C8-MPP2, or PSD-95 (see Fig. 2). Immunoprecipitations were performed using anti-SK2 antibody raised in guinea pig. Last three lanes show input material prior to immunoprecipitation. B) Co-immunoprecipitation of C8-SAP-97 co-expressed in HEK293 cells with GluA1 but not with myc-SK2-S. Western blot using anti-C8 antibody detects input of C8-SAP-97 co-expressed with myc-SK2-S or GluA1. C8-SAP-97 co-immunoprecipitated with anti-GluA1 antibody but not with anti-myc antibody or IgG. C) Western blot using anti-myc antibody detects myc-SK2-S co-expressed with C8-SAP-97, input and after immunprecipitation with anti-SK2 antibody. Higher MW bands correspond to aggregates of SK2-S. D) Western blot using mouse monoclonal anti-GluA1 antibody detects GluA1 co-expressed with C8-SAP-97, for GluA1 input and GluA1 immunoprecipitated with rabbit polyclonal anti-GluA1 antibody.

Supplementary Figure 3. GST-fusion protein expression.



Supplementary Figure 3. A) Coomassie stained gel showing input bacterial lysates (left lanes) for the indicated GST-fusion proteins prior to being bound to glutathione agarose beads, and after binding to beads (right lanes). B) Western blot of the gel in panel A, probed with anti-GST antibody.

Chapter III.

The role of SAP97 and MPP2 on the formation of a complex containing SK2 and AMPARs.

Introduction

As introduced earlier, SK2-containing channels are expressed in the PSD of CA1 pyramidal neurons and undergo LTP dependent endocytosis that requires PKA phosphorylation within the C-terminal domain of the SK2 subunit. Moreover, the endocytosis of synaptic SK2-containing channels requires the PKA dependent exocytosis of GluA1-containing AMPARs as a prerequisite step (Discussion). The coordinated dynamics of SK2 and AMPARs are precisely regulated and this suggests that molecular organization modulates synaptic distribution and trafficking. Chapter II demonstrated that MPP2 associates with SK2-containing channels and is required for synaptic localization and function of SK2-containing channels. In collaboration with Dr. Fakler, immuno-purification and proteomic studies using SK2 antibodies identified components of SK2-containing complexes in mouse brain. Tandem mass spectroscopy revealed SAP97, in addition to MPP2, is another candidate MAGUK interacting with native SK2. Moreover, SAP97 and MPP2 co-assemble with native AMPARs (Schwenk et al., 2012) suggesting that they may be capable of direct interaction and may be important for interdependent trafficking of SK2 and AMPARs. SAP97 and MPP2 contain L27 domains near their N-termini. L27 domains may mediate homo- or

heteromeric multimerization (Feng et al., 2004; Li et al., 2004).

These findings shape the further hypothesis: SAP97 and MPP2 may co-assemble with SK2 and AMPARs to control their coordinated trafficking in dendritic spines. To begin to address this, we first tested whether direct interaction exists between SK2 and SAP97 *in-vitro*, and if so, map the determinant domain of SAP97. The results have shown that SAP97 co-IP'd with SK2-L when co-expressed in HEK293 cells, and that L27 and SH3 domains are necessary for the interaction. Then we performed MPP2 co-IP with SAP97 from co-transfected HEK293 cells to assess direct interactions. The results showed that MPP2 co-assembles with SAP97. Further co-IP experiments revealed that GluA1 and MPP2 form molecular complexes, however, GluA1-containing AMPARs do not appear to form ternary complexes with SK2-containing channels via SAP97 and MPP2.

Results

SAP97 interacts with SK2-L isoform.

Quantitative proteomic analyses, performed in collaboration with Dr. Fakler, used SK2 antibodies to identify constituents of SK2-containing complexes from mouse brain membrane preparations. Tandem mass spectroscopy revealed SAP97 is a candidate MAGUK interacting with native SK2. To address whether SK2 directly interacts with SAP97, C8-SAP97 was co-expressed in HEK293 cells either with SK2-L with SK2-S or SK2-S only. Cell lysates were prepared for IP using anti-

SK2 antibody raised in guinea pig or IgG (control). Precipitated proteins were prepared as a Western blot and probed for C8-SAP97. The results show that SAP97 co-IP's when both SK2-L and SK2-S are co-expressed, but not with SK2-S only (Fig. 13). This indicates SAP97 interacts with SK2-L isoform.

Crucial role of L27 and SH3 domain of SAP97 for interaction with SK2-L

The co-IP experiment recapitulated the proteomics based on the finding of interactions between SAP97 and SK2 in brain (Fig. 14). To determine the domain of SAP97 crucial for interaction with SK2-L, deletion mutants of SAP97 were generated lacking either L27, PDZI, PDZII, PDZIII, SH3, or GK domains. SK2-L was co-expressed in HEK293 cells with the domain deletion mutants of C8-SAP97. Cell lysates were prepared for IP using anti-SK2 antibody or IgG (control), and Western blots of the IP material was probed for C8-SAP97. The Western blot showed that SK2-L could not interact with SAP97 lacking either the L27 or SH3 domains (Fig. 14).

SAP97 co-immunoprecipitates with MPP2

Both SAP97 and MPP2 coassemble with SK2-containing channels and native AMPARs (Schwenk et al., 2012), suggesting that they may be important for coordinated synaptic dynamics. SAP97 and MPP2 both include L27 domains that may mediate homo- or heteromeric formation with other L27 domain containing proteins (Nakagawa et al., 2004). To test whether SAP97 directly interacts with

MPP2, untagged SAP97 was co-expressed in HEK293 cells with C8-MPP2. IP was performed with anti-SAP97 antibody or IgG (control) and Western blots were probed for C8-MPP2. The results demonstrated that SAP97 co-immunoprecipitates with C8-MPP2 (Fig. 15).

GluA1 co-immunoprecipitates with MPP2

Proteomic analyses showed that native AMPARs co-immunopurified with MPP2 and SAP97 from mouse brain (Schwenk et al., 2012). In heterologous expression systems, SAP97 co-IP's with GluA1, and increases GluA1 surface expression and AMPAR-mediated currents (Waites et al., 2009). To examine whether MPP2 interaction with AMPAR is recapitulated *in-vitro*, GluA1 was co-expressed in HEK293 cells with C8-MPP2. IP was performed using anti-GluA1 antibody. The IP's were prepared as a Western blot and probed for C8-MPP2. Anti-C8 antibody detected co-IP'd MPP2 with equivalent level of IP'd GluA1 (Fig. 16). Thus, GluA1 co-assembles with MPP2 in a heterologous expression system.

SK2-containing channels do not form ternary complexes with GluA1-containing AMPARs via SAP97 and MPP2

Previous studies showed that AMPARs undergo LTP dependent exocytosis, which is required for SK2 endocytosis (Lin et al., 2010). This LTP-dependent trafficking of AMPARs and SK2 channels is interdependent and requires PKA activation. Previous experiments show that SAP97 and MPP2 interact each other

and can co-assemble with SK2-containing channels and AMPARs. This may indicate the existence of ternary complexes, composed of SAP97 and MPP2 that interact with AMPARs and SK2-containing channels. To test this possibility, SK2 and GluA1 were co-expressed in HEK293 cells together with either MPP2, SAP97 or both MPP2 and SAP97. Cell lysates were used for IP with anti-SK2 antibody and the Western blots were probed for GluA1. This failed to detect co-IP of SK2 and GluA1, suggesting that SK2 does not form ternary complex with GluA1 in any condition (Fig. 17A). The reverse co-IP was also performed using anti-GluA1 antibody and the blots were probed for SK2, and this also failed to detect interaction (Fig. 17B). One thing to note is, in both cases, co-expressed SAP97 and MPP2 co-IP'd with anti-SK2 or anti-GluA1 antibody (Fig. 16 bottom).

Conclusion

Co-IP experiments have shown that SAP97 directly interacts with SK2 in heterologous expression system. SAP97 contains multiple modular domains that mediate protein-protein interactions, consisting of a single L27 domain, three PDZ domains followed by SH3-HOOK-GK domains. Mutational co-IP study reveals that L27 and SH3 domains of SAP97 are crucial for SK2 interaction. Although SAP97 and MPP2 both coassemble with GluA1 or SK2, respectively, SK2 does not form ternary complex with GluA1 via SAP97 and MPP2 under the conditions tested.

Methods

Molecular biology

The cDNA sequence of SAP97 (Genebank accession number: NM_012788.1) from rat was used to construct deletion mutant of SAP97 lacking L27, PDZI, PDZII, PDZIII, SH3, or GK domain.

Co-immunoprecipitation

Forty eight hours post-transfection, cells were solubilized with lysis buffer (20mM HEPES, pH 7.5, 150mM NaCl, 10% glycerol, 2mM EDTA, 1mM PMSF, and protease inhibitors (Roche)) containing 1% β-D-dodecyl maltoside (DDM) (Sigma) for 30 min. The supernatant was incubated with guinea pig anti-SK2 antibody (C39) for 30 min then 20 ul of protein A/G agarose beads (Pierce) were added for incubation overnight at 4°C with rotation. The beads were washed 3 times in washing buffer (20mM HEPES, pH 7.5, 150mM NaCl, 10mM KCl, 10% glycerol, 2mM EDTA). Proteins were eluted with 40 ul of 2X SDS sample buffer at 37°C. Bound and eluted proteins were subsequently separated by SDS-PAGE and transferred to PVDF membrane. After blocking with 5% skim milk for 1 hour, membrane (Bio Rad) was probed with rabbit anti-SK2 antibody (Alomone) or mouse anti-C8 antibody (in-house) overnight at 4°C. HRP conjugated secondary antibodies for goat anti-rabbit or goat anti-mouse were applied for 30 min at RT. Blots were detected with SuperSignal ECL (Pierce) and developed with FILM BIOMAS LIGHT (Genemate).

Figure 13. SAP97 interacts with SK2-L isoform.

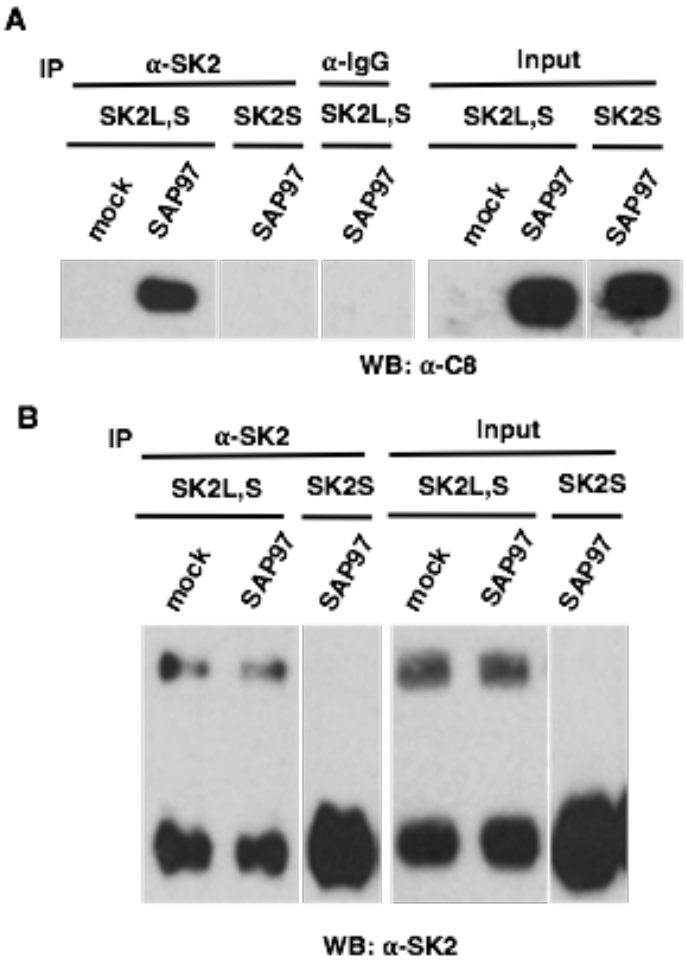


Figure 13. A) Co-immunoprecipitation and input of C8-SAP97 co-expressed in HEK293 cells with SK2-L, SK2-S or SK2-S only. Western blot using anti-C8 antibody detects co-IP'd SAP97 after IP with anti-SK2 antibody but not with IgG (control). B) Immunoprecipitation and input of SK2-L and SK2-S from transfected HEK293 cell lysates co-transfected with empty vector (mock) or C8-SAP97. IP's were performed using anti-SK2 antibody raised in guinea pig. Western blot was probed with anti-SK2 antibody raised in rabbit.

Figure 14. Role of L27 and SH3 domain of SAP97 for interaction with SK2-L

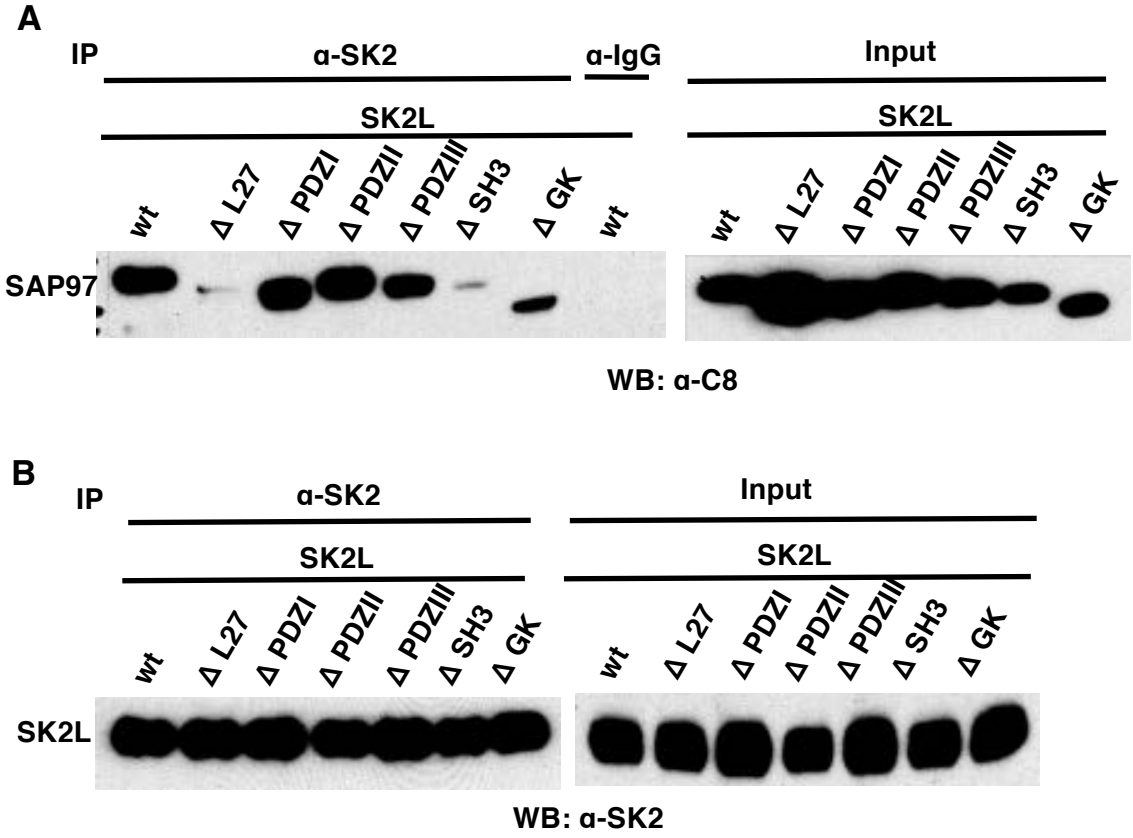


Figure 14. A) Co-immunoprecipitation and input of deletion mutants of SAP97 co-expressed in HEK293 cells with SK2-L. Co-IP of C8-SAP97 with anti-SK2 antibody is abrogated when L27 or SH3 domain are removed. B) Immunoprecipitation and input of SK2-L from transfected HEK293 cell lysates co-transfected with the deletion mutants of C8-SAP97. IP's were performed using anti-SK2 antibody raised in guinea pig. Western blot was probed with anti-SK2 antibody raised in rabbit.

Figure 15. SAP97 interacts with MPP2.

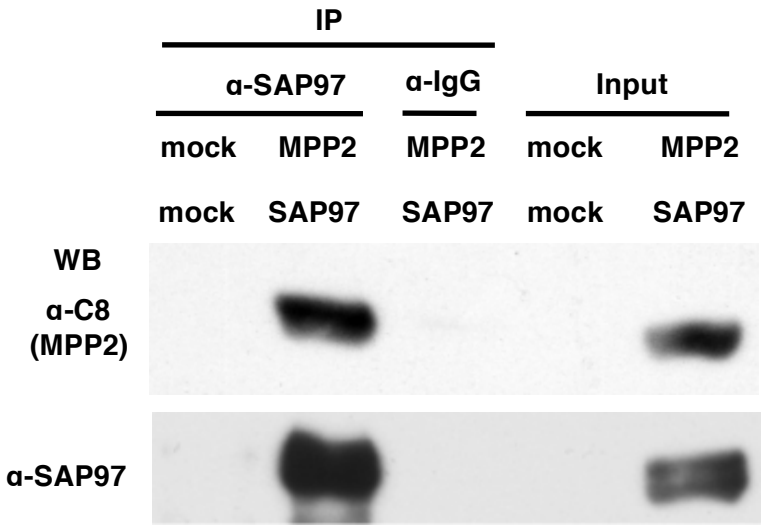


Figure 15. Co-immunoprecipitation and input of C8-MPP2 co-expressed in HEK293 cells with SAP97. C8-MPP2 co-IP'd with anti-SAP97 antibody but not with IgG. Western blot using anti-C8 antibody detects co-IP and input of C8-SAP-97. Bottom panel shows immunoprecipitation and input of SAP97 using mouse monoclonal anti-SAP97 antibody.

Figure 16. GluA1 interacts with MPP2.

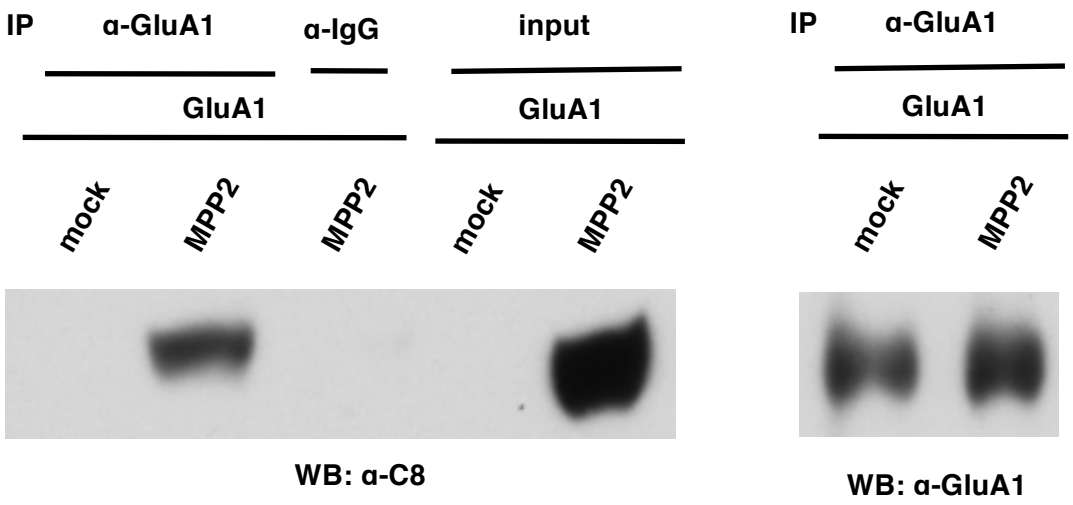


Figure 16. (left) Co-immunoprecipitation and input of C8-MPP2 co-expressed in HEK293 cells with GluA1. Western blots prepared from HEK293 cell lysates, mock transfected, expressing GluA1, or together with C8-MPP2. IP's were performed with either anti-GluA1 antibody or IgG (control), and the Western blot was probed for C8-MPP2. (right) Western blot shows immunoprecipitation and input of GluA1, probed with anti-GluA1 antibody.

Figure 17. SK2 does not coassemble with GluA1 via SAP97 and MPP2.

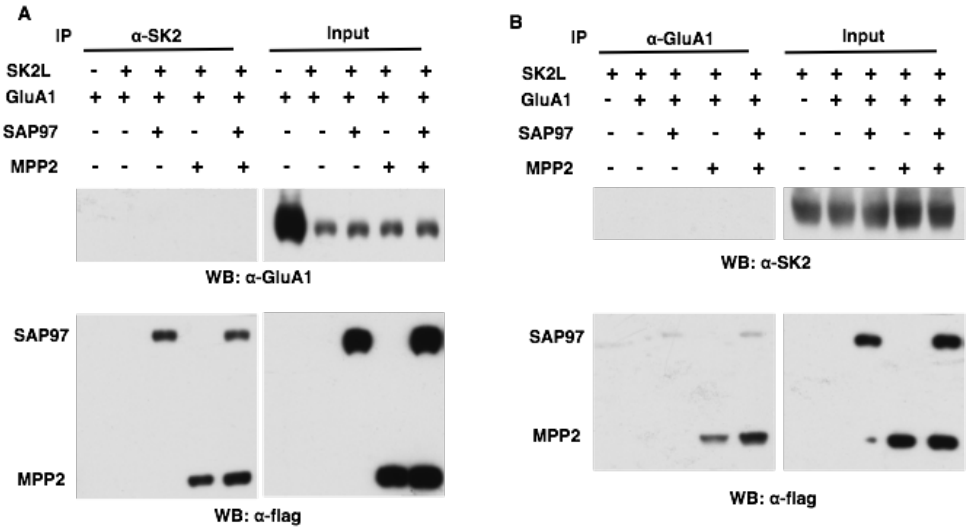


Figure 17. A) Western blots prepared from HEK293 cell lysates expressing GluA1, or GluA1 and SK2-L with either mock vector, flag-SAP97 or with flag-MPP2. Immunoprecipitations were performed with anti-SK2 antibody, and probed for GluA1 (upper), or flag-SAP97 and flag-MPP2 (bottom). B) Western blots prepared from HEK293 cell lysates expressing SK2-L, or SK2-L and GluA1 with either mock vector, flag-SAP97 or with flag-MPP2. Immunoprecipitations were performed with anti-GluA1 antibody, and probed for SK2 (upper), or flag-SAP97 and flag-MPP2 (bottom).

Chapter IV.

The influence of MPP2 knockdown on the NMDA:AMPA ratio

Introduction

Chapter II demonstrated that MPP2 associates with SK2-containing channels and is required for synaptic localization and function of SK2-containing channels. Biochemical studies revealed several binding partners of MPP2 in the PSD fraction of cortical neurons such as PSD-95, SAP97, GKAP, CASK, GRIP, neuroligin, and CaMKII (Jing-Ping et al., 2005). In addition, high resolution proteomics revealed that MPP2 co-assembles with native AMPARs from mouse brain (Schwenk et al., 2012). Chapter III further showed that MPP2 interacts with SAP97 and GluA1. This raises the question of whether knockdown of MPP2 expression may influence the synaptic distribution and function of AMPAR and NMDAR. To begin to test this, I measured AMPAR and NMDAR mediated EPSCs in control and MPP2 knockdown CA1 neurons. Increased NMDA:AMPA ratio was observed in MPP2 knockdown, with constant AMPAR component, indicating increased NMDAR contribution.

Result

To test whether MPP2 influences the NMDAR or AMPAR components of EPSCs, short hairpin RNAs targeting MPP2 were introduced into CA1 pyramidal neurons by IUE (E14~E16). Four weeks after birth, mice were used to prepare fresh hippocampal slices and EPSCs were recorded by stimulating SC synapses using

a Cs^{2+} based pipette solution. Cells were voltage clamped at a holding potential of -35 mV to relieve Mg^{2+} block on NMDARs. After establishing a stable baseline composed of both NMDAR and AMPAR EPSCs, D-AP5 (100 μM) was added to the bath solution to block NMDARs, and revealed the AMPAR component of the EPSC (Fig. 18A). The NMDAR EPSC was isolated after subtracting the AMPAR EPSC from the mixed EPSC (Fig. 18B). The total charge transfer of NMDAR component was measured as the integral at 50-100 ms after the synaptic stimulus, and used to calculate the NMDA:AMPA ratio. The scatter plot shows increased NMDA:AMPA ratio by MPP2 knockdown (Fig. 18C). This indicates that MPP2 knockdown may increase NMDAR component or decrease AMPAR component. To measure the pure AMPAR component, cells were clamped at -70 mV, effectively blocking NMDAR, and the total charge transfer of AMPAR EPSCs was measured at four different stimulating intensities (Istim) (Fig. 19A). Then the total charge transfer of AMPAR EPSCs was plotted as a function of the stimulating intensity (Istim). This yielded a slope and an extrapolated stimulus threshold. This calculated slope is primarily a measure of AMPAR component. The results showed that there was no significant change of stimulus threshold and slope of the EPSC Integral vs Istim between control and MPP2 knockdown cells (Fig. 19B, C).

Conclusion

MPP2 knockdown increased the ratio of total charge transfer between NMDAR and AMPAR EPSCs as shown by voltage clamp recording of EPSCs at -35 mV.

Since the increased NMDA:AMPA ratio might be due to either increased NMDAR or decreased AMPAR EPSC, we measured pure AMPAR component by plotting the stimulus intensity vs the AMPAR EPSC (Integral) (Fig. 19A). No change in the slope and stimulus threshold was observed (Fig. 19B, C), suggesting the AMPAR component is not altered in MPP2 knockdown cells. Further work will be required to understand just how the NMDAR component is affected by MPP2 knockdown. This is further discussed in the Discussion section, below.

Figure 18. The effect of MPP2 knockdown on NMDA:AMPA ratio.

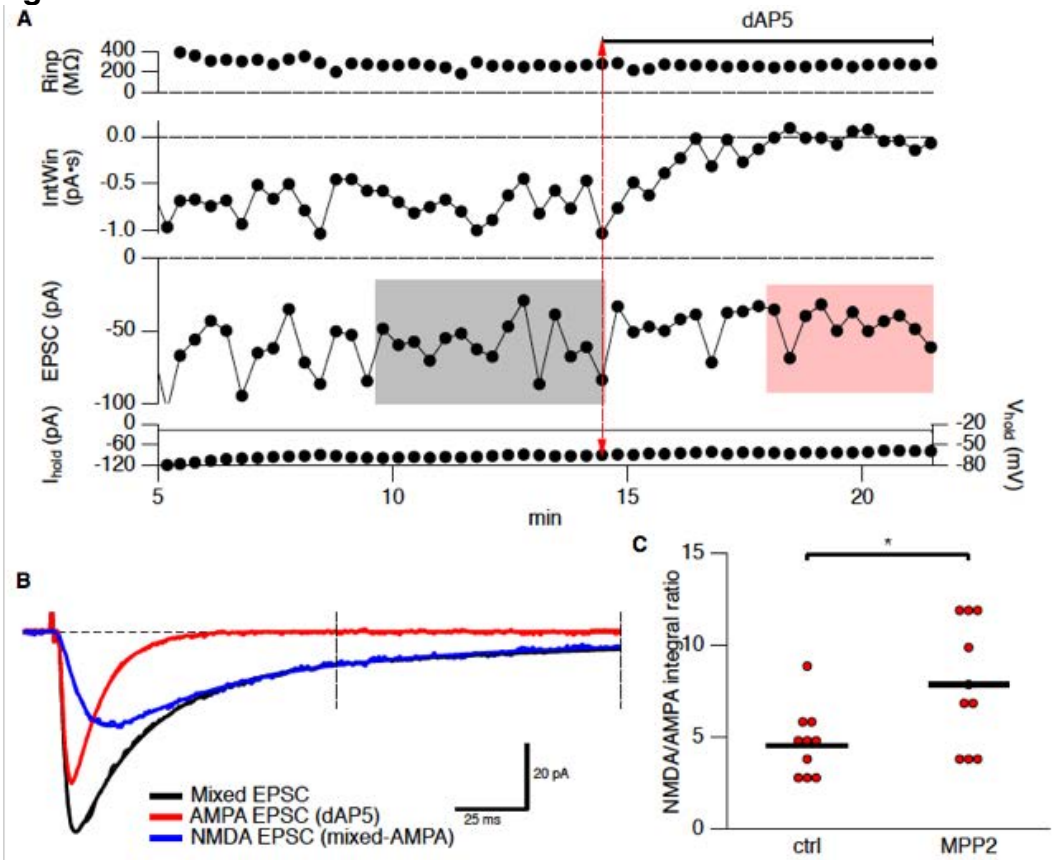


Figure 18. A) Diary plot of (top to bottom) input resistance (R_{inp}), charge transfer measured at 50-100 ms (IntWin) after stimulus (dash vertical lines in Panel B), EPSC amplitude (EPSC), and holding current (I_{hold}) from a representative cell during baseline and wash in of D-AP5 (100 µM). B) Average of 15 EPSCs acquired in baseline composed of mixed AMPAR and NMDAR EPSC (black), average of 10 EPSCs acquired in the presence of D-AP5 (red, AMPAR EPSC), and NMDAR EPSC after subtracting AMPAR EPSC from Mixed EPSC (blue). An exponential function ($Imax * \left(1 - e^{-\frac{(x-x0)}{tau_{on}}}\right)^3 * e^{-\frac{(x-x0)}{tau_{off}}}$) was applied to the AMPAR and NMDAR EPSCs (overlaid). C) Scatter plot of relative ratio of total charge transfer (measured from the fitted EPSC traces) between NMDAR and AMPAR EPSC in control cell (ctrl, n=10) and MPP2 knockdown cell (MPP2). Horizontal bar reflects mean response and asterisks reflects $p < 0.05$ determined using a nonparametric Wilcoxon-Mann-Whitney two-sample rank test.

Figure 19. AMPAR mediated EPSC is not affected by MPP2 knockdown.

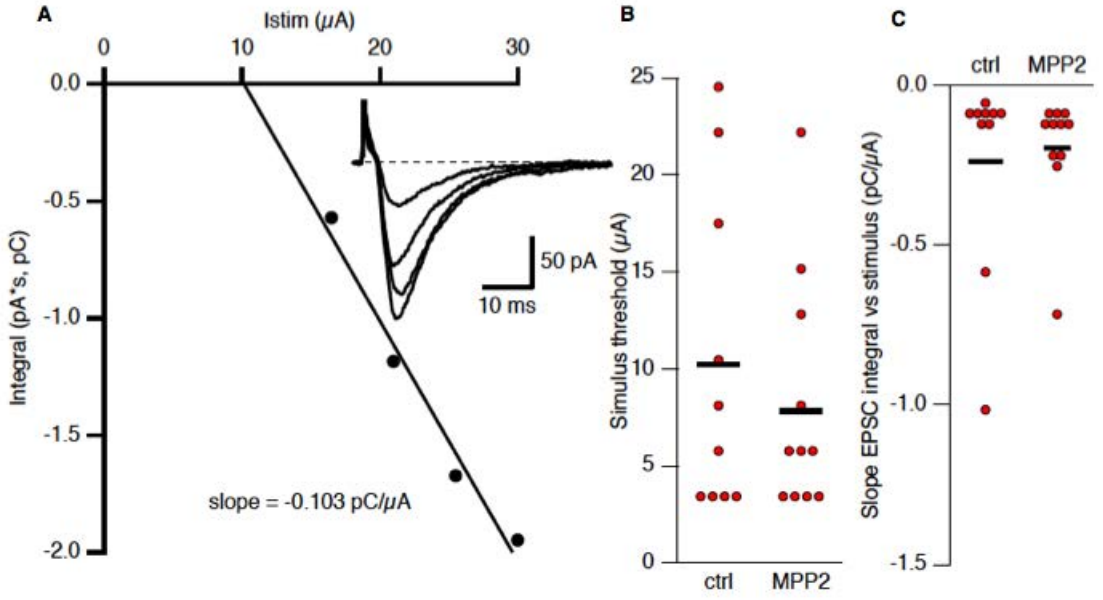


Figure 19. A) Example plot measuring total charge transfer of AMPAR EPSC (Integral) at different stimulating intensity (Istim). The plot of integral vs Istim is described by a linear relationship yield a slope of growth of AMPAR EPSC versus stimulus intensity (-0.103 pC/μA) and an extrapolated threshold 10.2 μA. Insets show AMPAR EPSC responses at four different stimulating intensities. B) Scatter plot of stimulus threshold in control cell (ctrl, n=10) and MPP2 knockdown cell (MPP2, n=10). C) Scatter plot of slope, calculated from (A), in control cells (ctrl, n=10) and MPP2 knockdown cells (MPP2, n=10). Horizontal bar reflects mean response.

Chapter V.

Testing whether synaptic SK2-containing channels require R-type Ca^{2+} channel activity

Introduction

The work from our laboratory has established that SK2-containing channels are expressed in the PSD of CA1 pyramidal neurons. Synaptic SK2-containing channels are activated by synaptically evoked Ca^{2+} influx that requires NMDARs activation (Ngo-Anh et al., 2005; Lin et al., 2008). This reduces EPSPs and spine Ca^{2+} transients by providing a repolarizing conductance that shunts the AMPAR mediated depolarization and attenuates unblock of NMDARs by external Mg^{2+}. Hence, blockade of synaptic SK2-containing channels with apamin increases EPSPs and Ca^{2+} transients in the spine (Ngo-Anh et al., 2005). Further Immuno-EM study demonstrated that NMDARs are co-localized with synaptic SK2-containing channels, and the occlusion of the apamin effect on EPSPs by NMDAR blocker and BAPTA, but not by EGTA, supports a nanodomain between synaptic SK2-containing channels and NMDARs (Lin et al., 2008). A subsequent study performed by another group provided a different model (Bloodgood and Sabatini, 2007). In this study, blocking R-type Ca^{2+} channels ($Ca_V2.3$) with SNX-482 (SNX) increased glutamate uncaging-evoked EPSP (uEPSP) and spine Ca^{2+} transients. This boosting effect of SNX occluded the effect of apamin on uEPSP and Ca^{2+} transients, suggesting $Ca_V2.3$ R-type channels serve as Ca^{2+} source for the activation of SK channels.

The ability of the NMDAR blocker D-AP5 to occlude the apamin effect on EPSPs supports the requirement for NMDAR activity, but in the model by Bloodgood and Sabatini, they postulate that NMDAR activity is required to boost spine depolarization to effectively activate R-type channels that provide the Ca^{2+} source for synaptic SK2-containing channel activation. More recently, our laboratory tested this hypothesis using synaptic stimulations. The results showed that the boosting effects of SNX and apamin were not mutually exclusive but additive, challenging the functional coupling between $Ca_V2.3$ and SK channels (Wang et al., 2014). This was further supported by the boosting effect of apamin on synaptically evoked EPSPs in $Ca_V2.3$ null mice, suggesting Ca^{2+} influx through $Ca_V2.3$ is independent from SK channel activation (Wang et al., 2015) and Ca^{2+} influx via R-type Ca^{2+} channels was proposed to serve as a distinct Ca^{2+} source for Kv4.2 containing channels activation mediated by their Ca^{2+} binding auxiliary subunits, KChiPs. Thus, there are two distinct Ca^{2+} microdomains within dendritic spines. One that couples Ca^{2+} influx through NMDARs to activate synaptic SK2-containing channels and one that couples Ca^{2+} influx through R-type Ca^{2+} channels to activate Kv4.2 channels.

The different conclusions appear to be due to the technical difference. The former result, which showed occlusion of apamin on EPSP by SNX, was obtained by 2-photon laser uncaging of glutamate with K^+ based internal solution (Bloodgood and Sabatini, 2007). K^+ based internal lacking Na^+ and Ca^{2+} channels blocker may cause more significant voltage escape than Cs^+ based

internal in voltage clamp configuration, particularly at -60 mV to adjust laser power for measuring EPSC that contains in part a postsynaptic outward current. As a result, SNX or apamin included bath condition may reduce a postsynaptic outward current when K^+ based internal is used in contrast to control bath condition. This may occur lower amount of glutamate uncaging in SNX or apamin included bath, and consequently lose its own control.

Given these contrasting results, our team scrutinized our own experiments. This raised the possibility that the flow rate of the bath solution in our experiments, ~1 ml/min, might be too slow and could result in ischemic changes to the slice during these prolonged experiments that requires multiple sequential drug applications. Moreover, the experiments had been performed by a single individual. Therefore, to reconcile these issues, I measured the effects of SNX and apamin on synaptically evoked EPSPs using a faster flow rate (2 ml/min).

Results

In current clamped CA1 pyramidal neurons, subthreshold EPSPs were measured with synaptic stimulation of SC synapses. In acute slices from wt mouse hippocampus, EPSPs were evoked in every 20 seconds using a flow rate of at least 2 ml/min. To determine whether the effects of apamin and SNX on EPSP are mutually exclusive, slices were pretreated with apamin (100 nM) and then a stable baseline was obtained. Then SNX (200 nM) or H_2O (control) was co-applied with apamin. SNX treatment boosted the peak EPSPs (148.7 ± 9.5%,

n=8, p = 0.003) in the presence of apamin, whereas H_2O treatment (control) did not affect EPSPs amplitude (96.4 ± 2.7%, n=5, p=0.26) (Fig. 20).

Conclusion

In this study, apamin pretreatment does not occlude SNX effect on EPSPs, supporting that Ca^{2+} influx through $Ca_V2.3$ is independent from SK channel activation, recapitulating and substantiating Wang's results.

Figure 20. The effects of SNX-482 and apamin on synaptically evoked EPSPs are not mutually exclusive.

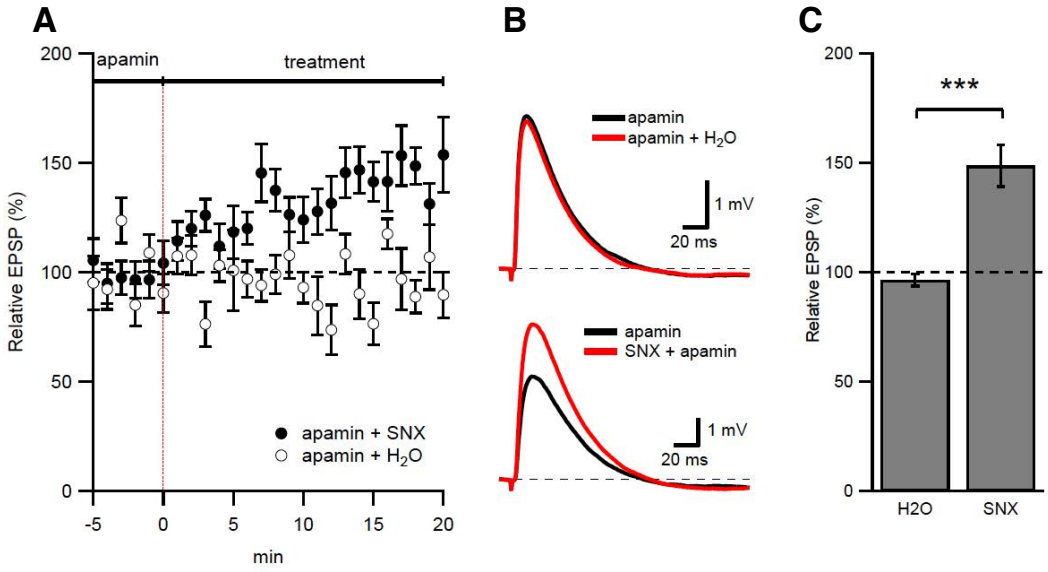


Figure 20. (A) Time course of the normalized EPSP amplitude (mean ± s.e.m.) for baseline in pretreatment of apamin and during wash-in of SNX (black symbol, n=8) and H_2O (open circle, n=5). (B) Average of 13 EPSPs taken from 5 min baseline in apamin and 15–20 min after co-application of SNX and H_2O (C) Bar graph of relative EPSP peak compared to baseline from co-application of H_2O and SNX in panel A. Horizontal bar reflects mean response.

Chapter VI. Discussion

Results and Summary

In hippocampal CA1 pyramidal neurons, SK2-containing channels are expressed in the PSD of dendritic spines, and are crucial components in synaptic transmission and plasticity. SK2-containing channels are functionally coupled to NMDARs that serve as the Ca^{2+} source for their activation (Ngo-Anh et al., 2005; Lin et al., 2008; Wang et al., 2014, 2015). Once activated, synaptic SK channels provide a repolarizing conductance that acts as a negative feedback to limit membrane depolarization and the spine Ca^{2+} transient by promoting Mg^{2+} block of NMDARs (Ngo-Anh et al., 2005). In addition, LTP induction results in endocytosis of synaptic SK2-containing channels that requires PKA phosphorylation near the C-terminus (Allen et al., 2010). This SK2 endocytosis is sequentially coordinated with PKA dependent exocytosis of GluA1-containing AMPARs as specifically blocking the exocytosis of GluA1-comtaining AMPAR also blocks the endocytosis of SK2-containing channel. However, the reverse is not the case (Lin et al., 2010). The endocytosis of synaptic SK2-containing channels contributes to the expression of LTP by reducing the repolarizing SK conductance. Thus synaptic SK2-containing channels have crucial roles in synaptic transmission and plasticity. These cellular roles are reflected in behavior as the modulation of SK channel activity influences hippocampal dependent memory encoding (Stackman et al., 2002; Allen et al., 2011).

The fundamental question of this thesis began with the observation that SK2-containing channels are preferentially localized in the PSD within the spine head of CA1 pyramidal neurons. Former studies showed that the additional 207 N-terminal amino acids present on the SK2-L isoform are required for synaptic localization and function of SK2-containing channels (Strassmaier et al., 2005; Allen et al., 2011). In the absence of SK2-L expression, SK2-S-containing channels were excluded from the synapse and distributed in the extrasynaptic membrane, and the boosting effect of apamin on EPSPs was absent. Reintroducing SK2-L recovered synaptic function of SK2-containing channels as shown by recovered apamin response on EPSP (Allen et al., 2011).

This raised the essential question, what is the underlying molecular mechanism that dictates synaptic localization of SK2-containing channels, and how does the unique N-terminal domain of SK2-L contribute to synaptic expression. The distribution of many synaptic receptors is determined by interactions with MAGUKs scaffold proteins. For example, PSD-95 uses PDZ interactions to stabilize synaptic NMDARs (Bassand et al., 1999). The trafficking and stabilization of AMPARs in the PSD is influenced by preferential interaction with TARPs, SAP97 and PSD-95 in an activity dependent manner, mediated by PDZ interactions (Chen et al., 2000; Tomita et al., 2005; Bats et al., 2007). Yet no MAGUK protein interacting with SK2-containing channels was known. The N-

terminal domain of SK2-L does not include a PDZ ligand but does contain a putative SH3 domain that typically interacts with proline-rich sequences.

MPP2 knockdown affects synaptic SK2-containing channels

The major finding of this thesis is that MPP2 is a novel MAGUK interacting with SK2-containing channels, which is required for synaptic localization. In collaboration with Dr. Fakler's laboratory, unbiased high-resolution proteomic analyses of SK2-containing channels immunoaffinity purified from rodent whole brain membrane preparations suggested that the MAGUK protein, MPP2 might be an interaction partner for SK2-containing channels. Therefore, the reverse purification was performed with highly specific, novel MPP2 antibodies, and clearly identified MPP2 as co-purifying with SK2. Further coIP following heterologous co-expression supports direct interactions between MPP2 and SK2. Specifically, MPP2 binds to N-terminal domain of SK2-L as shown by GST pull-down assays and it is the SH3-GK-HOOK domain of MPP2 that mediates interaction with the N-terminal domain of SK2-L. Immuno-EM shows that MPP2 is a synaptic MAGUK expressed in the PSD of CA1 pyramidal neurons. Knocking down MPP2 with MPP2 shRNA, introduced via in utero electroporation, abolished synaptic function of SK2-containing channels as shown by the absence of an apamin effect on EPSPs and reduced levels of LTP in hippocampal CA1 pyramidal neurons. Immuno-EM shows that MPP2 knockdown specifically reduces the synaptic population of SK2-containing channels, while the dendritic

population of SK2-containing channel is not obviously affected by MPP2 knockdown. SK2-containing channels in the proximal dendrites can be measured by an apamin sensitive tail current evoked by a somatic voltage pulse. Apamin sensitive currents were not altered by MPP2 knockdown. This suggests that MPP2 has a crucial role for synaptic recruitment and function of SK2-containing channels.

The role of MPP2 as a molecular hub

MPP2 contains various protein interaction domains including two L27 domains followed by a single PDZ, SH3, HOOK and GK domain. Previous biochemical assays showed the interaction of MPP2 with PSD-95, SAP97, GKAP, CASK, GRIP, neuroligin, cadherin, tubulin, actin, α-internexin, neurofilament-L and CaMKII via its PDZ, SH3, HOOK or GK domains (Jing-Ping et al., 2005). In this regard, the results from this thesis suggest the possible role of MPP2 as a molecular microdomain that regulates dynamics of its binding partners.

Upon the induction of LTP, the forward trafficking of GluA1-containing AMPARs is prerequisite for endocytosis of SK2-containing channels (Lin et al., 2010). This implicates the existence of molecular organization that regulates this coordinated trafficking of GluA1-containing AMPARs and SK2-containing channels for LTP expression.

In a previous study, MPP2 was immuno-purified with AMPARs from membrane preparations from rat brain (Schwenk et al., 2012). This was recapitulated by the

direct interaction of MPP2 with GluA1 in a heterologous expression system, HEK293 cells. Chapter II presents SAP97 as another binding partner of MPP2. SAP97 is a MAGUK protein that regulates AMPAR dynamics and was also identified in our unbiased proteomics study. Furthermore, unpublished results suggest that SAP97 may form a complex with SK2-containing channels. Indeed, Chapter III showed the direct interaction of SK2-L, but not with SK2-S, with SAP97, mediated by L27 and SH3 domains in a heterologous expression system. Each L27 and SH3 domain of SAP97 appear to be equally crucial for interaction with SK2-L, which explains the absence of interaction between N-terminal of SK2-L and SH3-HOOK-GK domain of SAP97 in a GST pull-down assay. Chapter III also demonstrated the direct interaction of SAP97 with MPP2 as implicated in the proteomics. This interaction is presumably due to L27 domain interactions that are known to mediate homo- or heteromeric multimerization. This aspect warrants further study.

Although the results from the thesis showed that SAP97 directly interacts with MPP2, and both SAP97 and MPP2 co-assemble with GluA1 and SK2, SK2 failed to form ternary complex with GluA1 mediated by MPP2 and SAP97. This raises several possibilities. One is that the scaffolds complex of SAP97 and MPP2 may not need to form ternary complexes with SK2 and may exist as separate pools for SK2-containing channels and GluA1-containing AMPARs in the basal condition. In this model, the discrete pools of scaffolded complexes may require additional

components to regulate the coordinated trafficking of SK2-containing channels and GluA1-containing AMPARs upon the induction of LTP. Second, SK2-containing channels and GluA1-containing AMPARs may compete for limited binding slots to interact with SAP97 and MPP2 in the PSD. Upon the induction of LTP, GluA1-containing AMPARs are exocytosed into the extrasynaptic membranes then translocated into the PSD (Yang et al., 2008) and stabilized by the interaction with SAP97, perhaps, MPP2. In this model, GluA1-containing AMPARs bind to empty slots or compete with pre-existing SK2-containing channels. In this scenario, PKA phosphorylation may confer higher affinity of GluA1-containing AMPARs for SAP97 and MPP2 over SK2-containing channels to exclude pre-bound SK2-containing channels from the scaffolds complex, which leads to endocytosis of SK2-containing channels during LTP. It will be interesting to know how the affinity of SK2-containing channels and GluA1-containing AMPARs for the scaffolds is different, and how this influences the coordinated trafficking in an activity dependent manner. Another possibility is that PKA phosphorylation of SK2-containing channels induces its endocytosis, then GluA1-containing AMPARs just occupy the binding slot of the scaffolds complex. Lastly additional PSD components may be needed in addition to the condition tested in Chapter III to form and stabilize ternary complexes of SK2-containing channels and GluA1-containing AMPARs with SAP97 and MPP2. Candidate components might be Lin7A and Lin7C as suggested as binding partner with MPP2 in Chapter II. MPP7, a closely related family member of MPP2, was shown

to interact with all family members of Lin7 (A, B, and C) mediated by L27 domains, in heterologous expression system (Bohl et al., 2007). This interaction stabilizes both components that lead to association with SAP97, which in turn influences the localization and stability of the triple complex. Therefore, it will be interesting to test whether Lin7A and Lin7C directly interact with MPP2, or what other components might be involved and how these additional components facilitate their functional coupling and stabilize synaptic organization.

Concern of shRNA for off-target effect

The main hypothesis of this thesis is that MPP2 has a crucial role on the synaptic function of SK2-containing channels. To test this, MPP2 shRNA was delivered to reduce MPP2 expression in hippocampal CA1 pyramidal neurons using in utero electroporation. Reducing MPP2 expression abolished the boosting effect of apamin on EPSPs, indicating the synaptic population of SK2-containing channels is diminished in the absence of MPP2. Importantly the reduced expression level in spines by MPP2 shRNA transfection was quantified on the level of single spines by immuno-EM. However, one concern using shRNA expression system was the possible off-target effects. This concern was overcome by co-expressing MPP2 shRNA (GFP) plasmid with shRNA-immune MPP2 (mApple) plasmid. Both GFP and mApple expressing CA1 pyramidal neurons were tested for rescue function of MPP2. Result showed that the apamin effect on EPSPs was recovered by shRNA-immune MPP2 co-expression, addressing the concern for

off-target effect and indicating that MPP2 shRNA specifically affects apamin sensitive SK2 containing channels in the synapse.

The contribution of MPP2 knockdown to LTP

One checkpoint is whether MPP2 knockdown may affect other synaptic components in addition to SK2-containing channels. Given the result that MPP2 has multiple binding partners, it is possible that MPP2 knockdown influences directly the stability and localization of binding partner, or indirectly other synaptic component that binds to the MPP2's binding partners. In Chapter II, the reduced expression of MPP2 by shRNA transfection decreased the level of LTP expression close to ~30%. However, previous study showed that the contribution of SK2 endocytosis to the LTP expression is close to ~17% (Lin et al., 2008). The larger effect of LTP expression may reflect effects of MPP2 knockdown on other PSD components. One explanation might be that MPP2 knockdown disrupts trafficking and stability of other scaffold protein, for example SAP97, which regulates synaptic trafficking of AMPARs. This may attenuate additional synaptic delivery or stabilization of AMPARs after LTP induction.

As discussed earlier, MPP2 knockdown might affect other PSD components, which in turn influence the response mediated by NMDARs and AMPARs. To assess this possibility, evoked EPSCs were recorded from CA1 pyramidal neurons at -35 mV. Then AMPAR mediated EPSCs were isolated after blocking

NMDARs with D-AP5 to calculate the ratio of NMDA:AMPA. Chapter IV demonstrated that MPP2 knockdown increased the NMDA:AMPA ratio, suggesting increased NMDAR and/or decreased AMPAR components. To dissect the pure AMPAR component, cells were voltage clamped at -70 mV to block NMDARs and the total charge transfer of AMPAR EPSCs was measured at four different stimulating intensities. The total charge transfer of AMPAR EPSCs was plotted as a function of the stimulating intensity to calculate the slope that represents primarily a measure of AMPAR component. This measurement showed no significant change in AMPAR component from MPP2 knockdown cells, which can be explained with two possibilities. One explanation is that the loss of SK2 by MPP2 knockdown simply attenuates the repolarizing K^+ conductance and extends the off time of Mg^{2+} from NMDARs that increases the total charge transfer via NMDARs, thereby increasing NMDA:AMPA ratio. Second possibility is that the MPP2 knockdown increased the NMDAR component, and the related question raised from the results is how MPP2 knockdown influences NMDAR component. PSD-95 is stably expressed in the PSD and interactions with NMDARs are crucial for NMDAR synaptic localization (Bassand et al., 1999; Chen et al., 2011). Therefore, it will be interesting to determine whether MPP2 knockdown affects synaptic expression of PSD-95 and NMDAR. It is also worthwhile to test which subtype of NMDAR may be preferentially susceptible to MPP2 knockdown.

Distinct Ca^{2+} microdomains in the dendritic spines

Our group showed that NMDARs forms a nanodomain with SK2-containing channels, and Ca^{2+} entry through NMDARs activates SK2-containing channels (Ngo-Anh et al., 2005). This is supported by the occlusion of the apamin effect on EPSPs by NMDAR blockers and BAPTA, but not by EGTA (Lin et al., 2008). Later, Sabatini showed that Ca^{2+} influx through NMDARs is not sufficient to trigger the activation of SK2-containing channels, but rather R-type Ca^{2+} channels selectively coupled to synaptic SK2-containing channels (Bloodgood and Sabatini, 2007). However, a follow up study from our group challenged the functional coupling between R-type Ca^{2+} channels and SK2-containing channels, and proposed rather that Ca^{2+} influx through CaV2.3 channels serves to activate Kv4.2-containing channels, acting via their Ca^{2+} binding auxiliary subunits, KChIPs (Wang et al., 2014). This was further supported in CaV2.3 null mice that showed a boosting effect of apamin on synaptically evoked EPSPs (Wang et al., 2015).

The different conclusions might arise from the technical differences, as Bloodgood and Sabitini used glutamate uncaging while our group employed synaptic stimulations. The experiments presented here were performed because of the importance of the fact that the two groups came to different conclusions, and so I repeated the central experiments in the Wang paper. My results showed that apamin pretreatment does not occlude the boosting effect of SNX on EPSPs

using synaptic stimulation and faster flow rate (2ml/min) than previously employed to avoid any possible source of error due to ischemic effects on the slices. This is consistent with Wang's conclusion and supports that R-type Ca^{2+} channels are a discrete Ca^{2+} source from SK2-containing channels. This demonstrates that R-type Ca^{2+} channels serve as a distinct Ca^{2+} source for spine transients compared to NMDAR, and Ca^{2+} entering the spine head through R-type Ca^{2+} channels does not fuel SK2-containing channels.

As discussed in Chapter V, glutamate uncaging method used in K^+ based internal appears to have considerably greater level of voltage escape than that of Cs^+ based internal where the postsynaptic outward current component contributes to the EPSCs in part. Therefore, different bath condition with SNX or apamin may elicit lower amount of glutamate uncaging compare to control solution, and consequently lose its own control. In contrast, synaptic stimulation does not precisely distinguish the location of spines being stimulated while the uncaging unambiguously defines the spines on first oblique branches within 100 μm of the soma. In addition, synaptic stimulation does not obtain any information about the nature of the stimulated spines while uncaging method chose mushroom type spines. Therefore, further work will be required to precisely evaluate the effect of each method.

Future Experiments

The major question raised from this study is the increased NMDA:AMPA ratio in basal condition by MPP2 knockdown. This might be just the consequence of decreased expression of synaptic SK2-containing channels by extending off time of Mg^{2+} from the NMDARs that increases the total charge transfer through NMDARs. However, finding an unaltered AMPA component suggests that this could be also due to increased NMDAR expression in the PSD. In order to investigate this, further work will be required to determine whether MPP2 knockdown affects PSD-95 or NMDAR synaptic expression. It will be also interesting to test if there is a preferential effect on NMDAR subtypes. Another interesting experiment to test is how the NMDA:AMPA ratio is influenced by MPP2 knockdown after induction of LTP. LTP induction leads to synaptic delivery of GluA1-containing AMPARs, hence expecting no change of NMDA:AMPA ratio in MPP2 knockdown cells.

The following experiment in the near future is characterizing MPP2 null mice. This MPP2 null mouse was generated and expanded for studying physiology and behavior. This will allow us to precisely dissect and understand the role of MPP2 on the orchestrated trafficking of synaptic MAGUK proteins, SK2-containing channels and ionotropic glutamate receptors, as well as on higher order functions such as learning and memory.

One of the goals in my thesis is studying the role of SAP97 on the SK2 physiology in CA1 pyramidal neurons. To follow this, knockdown method will be required to validate the functional role of SAP97. Since general knockout of SAP97 is embryonic lethal, conditional deletion or delivering of SAP97 shRNA will be a desirable approach. One concern in previous study reported the difficulty of knocking down of SAP97 and functional redundancy among other DLG family (Howard et al., 2010), while another study showed successful knockdown (Nakagawa et al., 2004). Therefore, careful validation of SAP97 expression will be necessary. One alternative is expressing dominant negative of SAP97 lacking SH3 or PDZ domain.

Conclusions

The results presented in this thesis demonstrate that the synaptic localization and function of SK2-containing channels is determined by MPP2 mediating through interactions with the N-terminal domain of SK2-L. MPP2 knockdown abolished synaptic function of SK2-containing channels in CA1 pyramidal neurons without affecting the dendritic population of SK2-containing channels. This tells us that the direct interaction between MPP2 and SK2 is crucial for the synaptic function of SK2-containing channels.

This study showed MPP2 expresses in the PSD of the hippocampal CA1 pyramidal neurons and suggests that MPP2 provides a molecular hub for PSD organization which might influence the dynamics and stabilization of its binding partners.

MPP2 contains multiple protein-protein interaction domains and co-assembles with SAP97, Lin7A, Lin7C and GluA1 in addition to SK2. SAP97 interacts with GluA1-containing AMPAR and also with SK2-containing channels mediated by L27 and SH3 domain. Therefore, this implicates the existence of molecular microdomains composed of MPP2 and SAP97 that regulates synaptic trafficking and stabilization of SK2-containing channels and GluA1-containing AMPAR. Given the reduced amount of LTP expression by MPP2 knockdown, it is reasonable to explain that the reduced amount of LTP expression by MPP2 knockdown is due to the combinatory effect of reduced expression of synaptic

SK2-containing channels and attenuated synaptic delivery and stabilization of GluA1-containing AMPAR by losing binding partner in the PSD of CA1 pyramidal neurons.

Working Model

Previously published findings and results obtained in this thesis suggest a working model as follows (Fig. 21). In the basal condition, SK2-containing channels are anchored in the PSD through molecular interactions with MPP2 and SAP97. MPP2 associates with SAP97 to engender a molecular hub mediated by L27 domains. The N-terminal domain of SK2-L is crucial for synaptic localization mediated by the interaction with MPP2 and SAP97. Synaptic localization of GluA1-containing AMPARs in steady state conditions is also mediated by the interaction with SAP97 and MPP2 via PDZ interactions. SK2-containing channels do not form ternary complex with GluA1-containing AMPARs but exist as discrete pool of MPP2-SAP97 complex, respectively.

LTP induction leads to trafficking of GluA1-containing AMPARs into the extrasynaptic membrane, then translocates into the PSD and competes with SK2-containing channels for MPP2-SAP97 interaction. LTP-dependent PKA phosphorylation endows higher binding affinity of GluA1-containing AMPAR over the limited binding slot of MPP2-SAP97. This expels SK2-containing channels from the molecular midcrodomain and leads to endocytosis from the synapse that contributes the expression of LTP. Another possibility is that PKA phosphorylation of SK2-containing channels causes them to be endocytosed from the synapse, then GluA1-containing AMPARs occupy the binding slot of MPP2-SAP97. This is consistent with the result that the reduced amount of LTP

expression in MPP2 knockdown cells (~30%) is larger than the contribution of the endocytosis of SK2-containing channels (~17%), which can be explained by the contribution of synaptic trafficking of GluA1-containing AMPARs.

Therefore, the molecular microdomain provides PKA access to GluA1-containing AMPARs and SK2-containing channels for their phosphorylation that leads to stabilized interaction of GluA1-containing AMPARs with the molecular microdomain, and endocytosis of SK2-containing channels from the synapse. This coordinated trafficking of GluA1-containing AMPARs and SK2-containing channels contribute the expression of LTP, which underlies hippocampus dependent learning and memory.

Figure 21. Schematic show of working model.

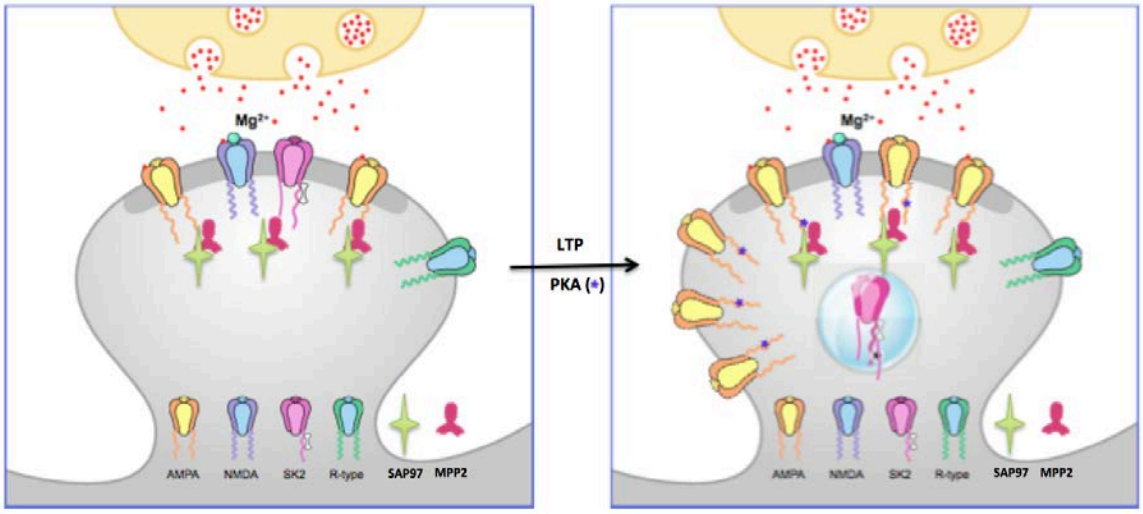


Figure 21. Left, under basal condition, the association between MPP2 and SAP97 forms a molecular hub in the PSD that recruits synpaptic localization of its binding partners such as SK2-containing channels and GluA1-containing AMPARs. In this steady state condition, SAP97 and MPP2 complexes serve as discrete pools to associate with SK2 channels or AMPARs, respectively. Due to the competition of SK2 and AMPARs to interact with SAP97-MPP2, they do not form a ternary complex with SAP97-MPP2 within the PSD. Right, upon the induction of LTP, PKA phosphorylates GluA1-containing AMPARs and increases their exocytosis into the perisynaptic membrane and translocation into the PSD. Increased synaptic trafficking of GluA1-containing AMPARs competes with SK2-containing channels that are already bound to SAP97-MPP2 complex. In addition, PKA phosphorylation at C-terminal domain of SK2-containing channels decreases the binding affinity with SAP97-MPP2 and leads to endocytosis from the synapse, allowing AMPARs to occupy the binding slot and associate with SAP97-MPP2, thus increasing synaptic strength.

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