Norepinephrine induces internalization of Kv1.1 in hippocampal neurons

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Abstract

Norepinephrine (NE), a catecholamine produced by dopamine β-hydroxylase, is released either as a hormone from the adrenal medulla into the blood, or as a neurotransmitter in the brain. Norepinephrine signaling serves as a global regulator of the brain, but there is still some functional selectivity in release and in the expression patterns of the various adrenergic receptor subtypes in different brain regions, to permit more specific sculpting of responses in the brain (O'Donnell et al., 2012).Extensive physiological and behavioral studies have demonstrated that NE/β2-adrenergic signaling could facilitate the induction of spike-timing-dependent plasticity (STDP) at excitatory synapses (Lin et al., 2003; Seol et al., 2007; Zhou et al., 2013) and enhances memory (Ji et al., 2003; Lemon et al., 2009; Miranda et al., 2011; Tronel et al., 2004), potentially through inhibiting dendritic potassium conductance and therefore increase the propagation of dendritic action potentials (Hoffman and Johnston, 1999; Watanabe et al., 2002). However, it remains unknown which potassium channels are involved in the modulation of dendritic excitability by NE/ β2-adrenergic signaling, and how these potassium channels are mobilized in response to β2-adrenergic receptor activation.

Our preliminary data showed that NE (norepinephrine) blocked the delayed onset of action potentials and induced the inactivation of D-type currents. This effect was mediated by signaling scaffolding SAP97, which belongs to the DLG-MAGUK family and has been indicated to regulate membrane expression and stabilization of PDZ-ligand-containing receptors and ion channels (Kim et al., 1995; Tiffany et al., 2000). A plausible mechanism would be the NE-induced reduction in surface expression of Kv1.1.

In this study, we showed that in primary hippocampus neurons, NE signaling, through β2 adrenergic receptor activity, induced internalization of endogenous Kv1.1. Additionally, the NE-induced internalization of Kv1.1 was impaired in SAP97 knockout neurons, suggesting that NE/β2-adrenergic receptor signaling can regulate surface Kv1.1 trafficking in a SAP97 dependent manner. Furthermore, we designed a PDZ/ligand class switch model by using compensatory mutants of SAP97 and Kv1.1, and characterized that the binding activity of SAP97 PDZ12 tandem was required for its interaction with the C-terminal PDZ domain binding motif of Kv1.1 and NE-mediated Kv1.1 internalization. Moreover, we found the PKA-mediated

phosphorylation of Kv1.1 was also responsible for the mobility of potassium channels in response to adrenergic signaling.

Overall, this study reveals a role of SAP97 in linking adrenergic signaling to the modulation of potassium channels surface expression, and provide a mechanistic understanding how G protein-coupled receptors regulate dendritic excitability.

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List of abbreviations

AC	adenylyl cyclase
AKAPs	A Kinase anchoring proteins
AMPAR	α-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid receptor
CACNB	Calcium channel β subunit
CARMA	Caspase recruitment domain proteins
CASK	Calcium/calmodulin-dependent protein kinase
CCPs	Clathrin-coated pits
CHO	cells Chinese Hamster Ovary cells
CME	Clathrin-mediated endocytosis
CRFR1	Corticotropin-releasing factor receptor 1
C-term	Carboxy terminals
EM	Electron microscopy
EPACs	Exchange Proteins directly activated by cAMP
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal regulated kinase 1/2
F-actin	Actin filaments
GABA	Gamma-aminobutyric acid
G-protein	Guanine nucleotide-binding regulatory
GRKs	G-protein coupled receptor kinase
GPCR	G-protein coupled receptors
HCN	Hyperpolarization-activated Cyclic Nucleotide-gated channels
hERG	Ether-à-go-go-Related Gene
HVA	High voltage activated calcium currents
ISO	Isoproterenol
Kv channels	Voltage-gated K+ channels

L27	LIN2-LIN7 domain
LC	Locus coeruleus
I_{Kur}	Ultra-rapid activating delayed rectifier potassium current
I_{to}	Cardiac transient outward potassium current
LTD	Long-term synaptic depression
LTP	Long-term synaptic potentiation
L-LTP	Late phase of LTP
MAGI	membrane-associated guanylate kinase with an inverted repeat
MAGUKs	Membrane-associated guanylate kinases
MiRP1	MinK-related peptide 1
NE	Norepinephrine
NMDAR	N-methyl-D-aspartate receptor
PDZ	PSD-95 Discs large Zona occludens 1 domain
PFC	Prefrontal cortex
PKA	Protein Kinase A
PSD	Postsynaptic density
SH3	Src homology 3
STDP	Spike-timing-dependent plasticity
TARPs	Transmembrane AMPAR regulating proteins
WM	Working memory
ZO	Zonula occludens proteins
4-AP	4-aminopyridine

1 Introduction

1.1 Potassium channels

Ion channels are integral membrane proteins that gate the flow of ions across the membrane through a small, water-filled pore. In prokaryotes, ion channels are critical for cell survival and growth, while in higher organisms, they become key components in a wide range of biological processes, particularly in the intercellular signaling communication between neurons.

In response to the appropriate stimulus, the cell membrane of a nerve cell goes through a sequence of changes, which is essential for neuronal communication. At the resting potential, voltage-gated ion channels are closed and ions could move through. However, when a stimulus is received by the dendrites of a nerve cell and the membrane is depolarized, voltage-gated Na+ channels open for a short period, allowing the influx of Na+ ions, which cause the depolarization associated with an action potential. Following the opening and closing of voltage-gated Na+ channels during an action potential, the transient opening of K+ channels causes the membrane potential to return to the resting state and even become more negative (hyperpolarization) for a short period, which is actually important for information transmission by preventing the neuron from receiving another stimulus during this time. The ability of axons conduct action potentials over long distances without diminution depends on controlled opening and closing of voltage gated Na+ and K+ channels (Uzman, 2001). In glutamatergic neurons in the hippocampus and cortex, there are at least three types of potassium currents involved inaction potential repolarization: IA (formed primarily by Kv4 family channels), ID (formed primarily by Kv1 family channels) and BK calcium-activated currents (formed by calcium-activated potassium channels) (Bean, 2007). Different K+ channel families are activated at different membrane potential and thus this determines whether they primarily repolarizing or hyperpolarizing.

Potassium channels (K+ channels) are the most diverse ion channel family, and the third largest group of mammalian signaling proteins (after GPCRs and protein kinases). The structural and functional diversity endows K+ channels variable roles in neurons.

1.1.1 Subunits composition

K+ channels contain principal subunits (α subunits), which determine the structure of the channel, and auxiliary subunits (β subunits), which modify the properties. K+ channels α subunits are multi-pass transmembrane polypeptides that contain the K+ selective pore, as well as the domains that allow them to respond to diverse stimuli. Most of the known α subunits express in heterologous expression systems as functional homo-multimeric channel complexes. Two-pore K+ channel α subunits assemble into dimers to function and all the others into tetramers. Some other α subunits co-assemble with auxiliary subunits for expression of functional channels.

K+ channel β-subunits tightly associate with pore-forming α subunits to regulate function and trafficking of the channel (Isom (1994). Some K+ channel β-subunits regulate inactivation, whereas others promote the maturation and cell surface localization of α subunits. For example, voltage-gated K+ channels are composed of ion-conducting integral membrane α subunits and cytoplasmic β-subunits, and it has been reported that the β-subunits affect the inactivation kinetics of α subunits, and therefore mediate the biosynthetic maturation and surface expression of voltage-gated K+ channel complexes (Shi et al., 1996). In addition, a molecular chaperone, KChAP, enhances the functional expression of a subset of Kv channels, such as Kv1.3, Kv2.1 and Kv4.3 in cardiomyocytes (Kuryshev et al., 2000). Some other examples include sulfonylurea receptors for the activity of ATP-sensitive K+ channels (Aguilar-Bryan et al., 1995; Jerng et al., 2004), MinK-related peptide 1 (MiRP1), a small integral membrane subunit, for the activation of HERG (also known as Kv11.1), which mediates the repolarizing IKr current in the cardiac action potential (Abbott et al., 1999), and distinct β-subunits for the functional properties of calcium-activated K+ channels (Brenner et al., 2000; Fajardo-Serrano et al., 2013; Klinger et al., 2011). These subunits play roles as diverse as regulation of gating properties such as activation/inactivation, membrane trafficking of ion channels, to serving as binding sites for both exogenous and endogenous ligands.

The subunit composition of K+ channels characterizes their intrinsic functions and properties, as well as diverse aspects of expression, localization, and modulation. As such, understanding the precise composition of K+ channels found at specific sites in mammalian neurons is crucial to define the basic tenants of their functions. Accurately defining the subunit composition of K+ channels also allows for the exploration of its pharmacological properties, which could be used to guide selection of in vitro and in vivo pharmacology models, which would then be used to

explore the consequences of channel modulation and further elucidate potential therapeutic utility (Rhodes and Trimmer, 2008).

1.1.2 Permeability (pore)

Potassium channels can selectively conduct potassium ions across membrane along its electrochemical gradient at a certain rate (106 to 108 ions per second). Potassium channels are endowed with several salient features in order to accomplish this: 1) a water-filled permeation pore which controls potassium ions movement; 2) a filter with selectivity that specifies potassium ions as its permeant ion species. 3) a gating mechanism which is able to switch open and closed (Hille, 2001).

The first gene encoded a potassium channel was cloned from Drosophila shaker mutant in 1987, indicating that the Shaker gene contains multiple exons distributed over at least 65 Kilobases of genomic DNA in the region where the mutations mapped, and these sequences supported the hypothesis that Shaker encodes a component of a potassium channel (Papazian et al., 1987). After that, more than 200 genes encoding a variety of potassium channels have been identified, all of which contain a highly conserved linker region connecting S5 and S6 transmembrane segments, a pole opening for potassium ions to flow passively across the membrane (Hartmann et al., 1991 Jurman et al. 1991).

Even though ion flow is fast, potassium channels are highly selective. Potassium channels share a highly conserved stretch of eight amino acids, a potassium channel signature sequence located in the S5-S6 linker, which is considered as the selectivity filter (Heginbotham et al., 1994). The first crystal structure of an ion-selective filter was reported by using the bacterial potassium channel KcsA as a model system (Doyle et al., 1998). At the resolution of 3.2 Å, the crystal structure of KcsA showed that the narrow selectivity filter is only 12 Å long, whereas the remainder of the pore is wider and has a relatively inert hydrophobic lining. Theses structural properties favor a high potassium throughput by minimizing the distance over which potassium ions interact strongly with the channel. On the basis of the structure, the filter is constrained in an optimal geometry so that a dehydrated potassium ion fits with proper coordination but the sodium ion is too small, and is thus poorly coordinated by the host.

A general classification of potassium channels into families is based upon the primary amino acid sequence of the pore-containing subunit: (1) 6TM/P channels, containing six trans-membrane helices and a single pore, which are the predominant class among ligand-gated and

voltage-gated potassium channels. (2) 2TM/P channels, containing two trans-membrane helices and a pore between them, exemplified by inwardly rectifying K+ channels and bacterial K+ channels such as KcsA. (3) 4TM/2P channels, which consist of two repeats of 2TM/P channels. (4) 8TM/2P channels, which are hybrids of 6TM/P and 2TM/P. Two-pore KCh α subunits assemble into dimers to function, and all of the others into tetramers. Auxiliary subunits generally coassemble with these tetrameric α subunits complexes in a subbranches specific manner.

1.1.3 Voltage-gated K+ channels (Kv channels)

Kv channels are the largest group of the K+ channel family, which also represents the most diverse repertoire of ion channels expressed in the nervous system. Due to the diversity of Kv channel α subunit genes, molecular characterization of Kv channel originally lagged behind that of Nav and Cav channels. The breakthrough of Kv channel molecular characterization came from the cloning of Drosophila shaker potassium channels (Papazian et al., 1987), which leads to the cloning of the mammalian orthologue Kv1.1. From the deduced shaker amino acid sequence, it was found that α subunits of Kv channels strongly resembled one of the four internally repeated homologous pseudo subunit domains of an Nav or Cav channel. This data resulted in the proposal which was initially substantiated by biophysical studies and further proved by the visualization of cryo-electron microscopic structure, that Kv channels are comprised of tetramers of α subunits(MacKinnon; Misonou et al., 2006; Riazanski et al., 2001; Sokolova et al., 2001).

Kv channels share a common design of four α subunits, which could be separate proteins that assemble in the endoplasmic reticulum or strung together into a large polypeptide. Each α subunits contains six trans-membrane helices, S1-S6, which are arranged circumferentially around a central pore as homo-tetramers or hetero-tetramers. S1-S4 form the voltage sensing domains (one per each subunit) with the highly charged S4 being the main component. S5 and S6 helices of each subunit come together to form the pore-lining domain as a selectivity filter for potassium (Grunnet et al., 2014; Lemaillet et al., 2003).

In human, there are twelve Kv channel subfamilies which are encoded by 40 genes, some of which can generate messages that are subject to alternative splicing. Besides the great variety

of genes and splicing isoforms of Kv channel, Kv channel also shows differential regional and subcellular localization in the CNS.

1.1.4 Subcellular localization of K+ channels

In mammalian central neurons, Kv channels exhibit specific expression patterns with subcellular segregation. Kv1 channels are predominantly localized in axons and β subunits play a prominent role in determining this localization. It has been shown that Kv β subunits, which enhance cell surface expression of Kv1.2 in COS-1 cells, can also induce the targeting of Kv1.2 to axons in cultured hippocampal neurons, recapitulating the subcellular localization of Kv1.2 observed in mammalian central and peripheral neurons (Campomanes et al., 2002; Trimmer, 2015). Subsequent research showed that the intracellular T1 domain of the Kv1 α subunit is essential for its interaction with β subunits and axonal expression (Grunnet et al., 2014; Gu and Barry, 2011; Gulbis et al., 2000; Hoffman, 2013).

Although the rules for Kv channel assembly are relatively simple, the determinants defending which subunit combination traffic through the endomembrane system to the plasma membrane are more complex. It has been shown in heterologous expression systems that Kv1 α and β subunits can assemble promiscuously into homo- and heterotetrameric complexes yielding a wide variety of biophysically and pharmacologically distinct channels (Rettig et al., 1994) (Elezgarai et al., 2003), while biochemical studies show that only specific Kv1 heteromeric complexes predominate in mammalian brain and other possible subunit combinations are not detected (Hanner et al., 1999; Lorincz and Nusser, 2010; Poolos and Johnston, 2012). Further studies showed dramatic differences in trafficking, biosynthetic processing and surface expression among these subunit combinations, and the surface trafficking of Kv1 channels is promoted by the coassembly with Kvβ subunit (Child and Benarroch, 2014; Trimmer, 2015). These observations suggest not only a functional importance for particular heteromeric K+ channel complexes, but also cellular mechanisms exist to restrict surface expression to certain channels with appropriate subunit composition. However, the mechanisms that regulate cell surface expression of K+ channels remain to be elucidated.

The expression of K+ channels are extremely diverse due to multiple gene families, heteromultimeric combination of α and β subunits, splicing variants and post-translation regulation, but Kv1 family are also richly localized to somatodendritic compartments (Guan et al., 2006) (Manganas and Trimmer, 2000). Single cell RT-PCR suggested in individual layer II/III

pyramidal cells, Kv1.1 is the most abundant subunit among Kv1 family on mRNA level (Guan et al., 2006; Howell et al., 2010; Sarmiere et al., 2008). It has been shown that Kv1.1 could be locally synthesized in dendrites, and Kv1.1 local translation could be regulated by NMDA-dependent synaptic activity (Raab-Graham et al., 2006; Watanabe et al., 2002). Another study characterized the dynamic regulation of dendritic expression of Kv1.1 in hippocampus during associative learning. Kv1.1 mRNA level is transiently down-regulated in the early stage of learning, suggesting the modulation of Kv1.1 expression is crucial for the regulation of neuronal substrates underlying new information acquisition (Kourrich et al., 2005). Structural and functional assays identified the C-terminus of Kv1.1 could interact with the PDZ domains of MAGUK family scaffold protein SAP97, which were thought to regulate the membrane trafficking and stabilization of PDZ-ligand-containing protein, such as potassium channels and adrenergic receptors (Kim et al., 1995; Tiffany et al., 2000) (Jan and Jan, 2012).

1.2 Adrenergic receptors

Norepinephrine (NE), a catecholamine produced by dopamine β-hydroxylase, is released either as a hormone from the adrenal medulla into the blood, or as a neurotransmitter in the brain. A majority of brain noradrenergic neurons are concentrated in the brainstem nucleus, locus coeruleus (LC), which is the sole source of NE to hippocampus and neocortex, regions critical for higher cognitive and affective processes (Berridge and Waterhouse, 2003). LC is a small potine nucleus composed of approximately 1500 noradrenergic neurons, which locate within the dorsal wall of the rostral pons in the lateral floor of the fourth ventricle and have broad projections spreading throughout the central nervous system, with heavy innervation of the spinal cord, brain stem, cerebellum, amygdala, hypothalamus, thalamic relay nuclei, cingulate gyrus, striatum, basal telencephalon, cortex and hippocampus (Loizou, 1969). Early studies showed that these projections are primarily composed of non-junctional varicosities, which may release norepinephrine into the extracellular space and reach nearby receptors by molecular diffusion (Séguéla et al., 1990). As a result, norepinephrine signaling from LC serves as a global regulator of the brain, but there is still some functional selectivity in release and in the expression patterns of the various adrenergic receptor subtypes in different brain regions, to permit more specific sculpting of responses in the brain (O'Donnell et al., 2012).

1.2.1 NE neurons in the LC nucleus

LC can directly modulate the somatosensory information from peripheral systems and e.g. completely inhibit the input from painful stimuli through the descending projection to the spinal cord under stress conditions (Stahl and Briley, 2004). Electrophysiological studies have shown that LC neurons are highly plastic and showed rapid plasticity in response to stimuli that drive their activation (Bouret and Sara, 2004). Several lines of evidence suggested that dysregulation of LC neurotransmission is involved in physical painful symptoms, sleep/arousal disorders, schizophrenia, attention deficit hyperactivity and Parkinson's disease (Huang et al., 2012).

Traditionally investigators have suggested that the LC-NE system serves relatively simple and basic functions (Berridge and Waterhouse, 2003), but recent studies revealed LC neurons in LC nucleus exhibit two modes of activity corresponding to different patterns of performance: phasic and tonic. In the phasic mode, LC cells display phasic activation driven by the processing of task-related stimuli but exhibit only a moderate level of tonic discharge, which is proposed to facilitate ensuring behaviors and to help optimize task performance (exploitation). This mode of function is consistent with high levels of task performance. When utility in the task wanes, these neurons fail to respond phasically to task events but exhibit a tonic activity mode, associated with disengagement from the current task and a search for alternative behaviors (exploration). This mode is associated with poor performance on tasks that require focused attention and corresponds to apparent increase in distractibility (Aston-Jones and Cohen, 2005). As such, NE neurons in the LC nucleus have the potential to alter the neuronal responses both to slow changes in physiological function and critical moments of task-related stimuli.

1.2.2 Modulation of excitatory neuronal responses and synaptic plasticity

Hippocampus receives abundant innervation from noradrenergic afferents from LC and is considered to be an important locus of the NE actions linked to the mechanisms of learning and memory. Early studies have shown that NE could be released in hippocampus in an activity-dependent way, and the application of NMDA can increase the release of NE in both dentate gyrus and hippocampus (Andrés et al., 1993). It has also been shown that NE, through β adrenergic receptors, and other neuromodulators could limit spike frequency adaptation and increase transmission of action potential trains in hippocampal pyramidal neurons, and this modulation is through the suppression of Ca^{2+}-activated K^+ current I_{AHP} and mediated by Protein Kinase A (PKA) (Pedarzani and Storm, 1993). Activation of β adrenergic receptors is involved in

enhancement of memory after emotional experiences, induces long-lasting strength in the perforant pathway (Dahl and Sarvey, 1989). Electrophysiological studies suggested that β-adrenergic receptor activation was paired with the late phase of LTP (L-LTP) induced by subthreshold synaptic stimulation on hippocampal slices , and this L-LTP required protein synthesis but not transcription (Gelinas and Nguyen, 2005). Another study in hippocampus demonstrated that NE could induce the phosphorylation of GluA1-containing AMPARs at sites Ser845 and Ser831, which are critical for its synaptic trafficking, and this effect is necessary to lower the threshold for GluA1 synaptic incorporation during LTP and for memory formation in behavioral experiments (Hu et al., 2007). Moreover, phospho-mutant mice with knock-in mutations on these sites have similar defects in NE-facilitated LTP and NE-enhanced contextual memory tasks. These data indicates that in hippocampus, NE plays an important role in synaptic receptor trafficking during the emotional regulation of learning and memory.

Electrophysiological and cognitive experiments showed that in prefrontal cortical (PFC) neurons, NE could activate α2 adrenergic receptors, inhibit the production of cAMP, close Hyperpolarization-activated Cyclic Nucleotide-gated channels (HCN), a type of non-selective cation channels, strengthen the functional connectivity of PFC networks, and therefore improve spatial working memory (WM) (Wang et al., 2007). This α2-AR-cAMP-HCN signaling may provide a mechanism for dynamically regulating the strength of PFC networks, and for determining of which pattern of microcircuits are functionally connected at any one time to appropriately regulate behavior and thought based on immediate cognitive demands.

However, NE is also involved in decreasing excitatory transmission. Activation of α2 adrenergic receptors by NE decreased high voltage activated (HVA) calcium currents ($Ca_v2.2$, N-type calcium channels) in a concentration-dependent, reversible manner, and this modulation was mediated by PKC-mediated phosphorylation of α2 adrenergic receptor's G-protein's βγ subunit binding site (Timmons et al., 2004 2005). Early in vivo studies have shown that application of NE and isoproterenol (ISO) can enhance gamma-aminobutyric acid (GABA)-induced depressant responses of rat somatosensory cortical neurons, thereby sensitizing cortical networks to inhibitory input (Sessler et al., 1995).

1.2.3 Adrenergic receptors

All adrenergic receptors belong to the guanine nucleotide-binding G protein-coupled receptor (GPCR) family, which is the largest group of membrane-expression receptors in mammals and comprises more than 1% of the human genome (Fredriksson et al., 2003). One of the defining feature of the GPCR superfamily is that all of the receptors bind to heterotrimeric guanine nucleotide-binding regulatory protein (G-protein). GPCRs share a conserved structure of seven transmembrane α-helices forming three extracellular loops, with one being the amino-terminus, and three intracellular loops, including the carboxy-terminus (Johnson, 2006). The G protein βγ subunits ($G_{\beta\gamma}$) form a tight dimer which is bound to the intracellular plasma membrane via an isoprenyl moiety located on the carboxy-terminus of the γ-subunit. Upon binding of a ligand to GPCR, GDP is released from G_{α}, and subsequent GTP binding occurs, which activates the G_{α} subunit and also exposing effector-interaction sites in $G_{\beta\gamma.}$ There are four main families of G_{α} subunits based on theirs primary sequence: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q/11}$ and $G_{\alpha 12/13}$, which regulate the activity of many different second messenger systems (Lynch and Ryall, 2008). The most well-characterized family of GPCRs belongs to the rhodopsin subfamily, which include dopaminergic, adenosine, histamine, and adrenergic receptors.

There are two main groups of adrenergic receptors, α and β, with several subtypes. The subtype of α adrenergic receptors includes $G_{\alpha q/11}$-coupled α1 receptors and $G_{\alpha i}$-coupled α2 receptors. β adrenergic receptors have the subtypes β1, β2 and β3, linked to $G_{\alpha s}$ proteins, which in turn are linked to adenylate cyclase. Activation of β receptors by agonist binding typically causes a rise of intracellular concentration of the second messenger cAMP. Downstream effectors of cAMP include cAMP-dependent kinase PKA, which can mediate some intracellular events following hormone binding. α adrenergic receptors can be activated by catecholamine either via neural synapses or the circulation, and mediate a diverse range of physiological events. The activity of the α1 adrenergic receptor is involved in the vasoconstriction of large resistance arterioles, regulation of blood pressure and cardiac adaptation to stress, while the α2 adrenergic receptor plays an important role in neurotransmitter release, cardiovascular performance, and the response to sedation, anesthetics or analgesics (Lynch and Ryall, 2008).

β adrenergic receptors are widely studied due to their regulatory roles in cardiovascular, metabolic, respiratory and reproductive function (Gardner et al., 2011; Lynch and Ryall, 2008). β1 adrenergic receptor (β1-AR) is found at its highest levels in the heart and brain, β2 adrenergic receptor (β2-AR) is relatively more widely expressed, and β3 adrenergic receptor

(β3-AR) is mainly found in adipose tissue. The β2-AR plays a critical role in mobilizing energy stores and redirecting blood flow during the fight-or flight response, which is an evolutionary conserved response that is activated upon encounter of a stressful stimulus, and expresses on multiple cell types involved in immune regulation, including immune cells and non-immune cells associated with immune response (Kolmus et al., 2015; Tran et al., 2007). The activation of β2-ARs initiates signaling through either a heterotrimeric G_s protein or β-arrestin, resulting in an enhancement of adenylyl cyclase (AC) activity, which converts ATP to cyclic AMP (Campomanes et al.). cAMP serves as a second messenger, which activates either protein kinase A (PKA) or the guanine Exchange Proteins directly activated by cAMP (EPACs). PKA is considered to be the main target for cAMP in the cell and without activation, PKA stays as an inactive tetrameric holoenzyme consisting of two catalytic subunits bound to a regulatory subunit dimer, which is compartmentalized to distinct locations in the cell by A Kinase anchoring proteins (AKAPs) (Keil et al., 2016). PKA phosphorylates proteins that have the motif Arginine-Arginine-X-Serine exposed, resulting in activation or deactivation of the proteins involved in several important functions in the cell, including the regulation of metabolism and triglyceride storage. It has been shown that β2-ARs could also couple to G_i protein in some types of cells, and in this context, PKA-mediated phosphorylation plays a key role to switch coupling of β2-AR from G_s to G_i protein and hence reversing the G_s protein-mediated generation of cAMP (Daaka et al., 1997; Hall et al., 1998). In addition, β-arrestin is also found to bind and desensitize the activated and phosphorylated form of the β2-AR (Benovic et al., 1987), and functions as a signal transducer, bringing β2-ARs into multiple signaling pathways, such MAPK, ERK and NF-κB (Lefkowitz et al., 2006).

1.2.4 GPCR membrane trafficking in the endocytic pathway

GPCRs are subject to exquisite regulation by the coordinated actions of multiple mechanisms. One general class of modulatory mechanisms is through posttranslational modification, such as phosphorylation, ubiquitination and acylation, which can produce a wide array of effects on the ability of receptors to bind their ligands and to interact with different downstream regulators and mediators in the cytoplasm. Another class of mechanisms is through receptor trafficking from one membrane compartment or subdomain to another (Hanyaloglu and von Zastrow, 2008; Marchese et al., 2008). The physical movement of GPCR can directly modify cellular signaling responsiveness by dynamically changing the number of functional molecules, which are

potentially activated by neurotransmitters in target neurons or in a specific type of neurons with particular subcellular localizations in the brain (Cao et al., 1999; Maxfield and McGraw, 2004; Puthenveedu et al., 2010; von Zastrow and Williams, 2012). (Cao et al., 1999)

It has been shown that GPCRs engage into multiple endocytic mechanisms, and the majority of them can be internalized by clathrin-mediated endocytosis (CME), which is a versatile endocytic pathway for many different cargoes to be packed using a range of accessory adaptor proteins. CME is considered to be fundamental to neurotransmission, signal transduction and regulation of many plasma membrane activities and is thus essential to higher eukaryotic life (McMahon and Boucrot, 2011). In most of the cases, GPCRs are phosphorylated by G protein-coupled receptor kinase (GRKs), which discriminates between inactive and agonist-activated states of the receptors, leading to enhanced association with β-arrestins, with concomitant attenuation of G protein-dependent signaling (Ferreira et al., 2012). The phosphorylation-dependent β-arrestin-binding uncouples the receptors from G protein by steric hindrance and targets the receptor to clathrin-coated pits (CCPs) through the binding of AP-2 and clathrin. Recent studies have shown that β-arrestins are primarily localized in the cytoplasm and interact with GPCRs, followed by activation and phosphorylation by GRKs, and thereby mediate many signaling events (Moore et al., 2007). However, GPCRs can also traffic into CCPs in an arrestin-independent manner, in which after being delivered to endosomes, GPCRs are rapidly recycled for resensitization or targeted to lysosomes for degradation (Premont and Gainetdinov, 2007).

In vivo, a large number of GPCRs undergo internalization induced by agonist-activation, but it is still unclear about the condition under which it occurs. In the case of β2-ARs, agonist-activated receptor internalization not only contributes to receptor desensitization but also induces receptor dephosphorylation. The internalization of β2-ARs also requires the binding of β-arrestin, which functions as an intermediary endocytic adaptor to recruit β-adaptin and thus target the receptor to CCPs for endocytosis (Goodman et al., 1996). Then internalized β2-ARs are either directly transported to lysosomes for degradation, or recycled back to the plasma membrane for resensitization. In general, β2-AR signal transduction is based on a coordinated balance between processes governing agonist-activation, desensitization and resensitization (Seachrist et al., 2000). But regarding other β-subtypes of adrenergic receptors (β1 and β3-AR), unlike β2-AR, β3-AR does not internalize in response to agonist-binding; likewise, compared to β2-AR, human β1-AR is more resistant to agonist-activated down-regulation and the internalized β1-AR is sorted to different endosomal compartments, even though it shares a similar endocytic mechanism with β2-ARs (Di Certo et al., 2008).

1.3 Membrane associated guanylate kinases

In the central nervous system, GPCR and ion channels are primarily targeted to the membrane of dendritic postsynaptic terminals of excitatory synapses in and around a region called the postsynaptic density (PSD), each of which is specifically organized, so that dozens to hundreds of membrane-spanning neurotransmitter receptors and submembrane-thickening enriched signaling enzymes and cytoskeletal proteins are located on this specialized membrane domain via the interaction of scaffold proteins (Dunn and Ferguson, 2015). The scaffold proteins of the PSD are composed of multiple functional domains, which mediate interactions among synaptic proteins and thus hold them in close proximity with each other and allow them to interact with a multitude of structural and signaling proteins (Feng and Zhang, 2009). MAGUKs, among these synaptic scaffold proteins, are believed to be the most abundant and often provide direct contact with both ion channels and GPCRs at the PSD via their PDZ domains (Feng and Zhang, 2009).

1.3.1 The postsynaptic density and MAGUKs

Early observation with electron microscopy (EM) discovered an electron-dense thickening (30-50 nm) located beneath the postsynaptic membrane (Palay, 1956), which is later been termed as PSD and identified by EM and biochemistry analysis as a protein aggregate composed of thousands of different proteins with a wide range of abundances, including neurotransmitter receptors, cell adhesion and signaling molecules, cytoskeleton proteins and scaffold proteins, as well as protein synthesis and degradation machineries (Zhu et al., 2016). PSD proteins are linked to actin filaments (F-actin), which is the main structure framework of the spine and a key modulatory site for plasticity (Bosch et al., 2014). In response to different neuronal stimuli, the structure of the PSD is highly plastic in both developmental and mature neurons, and the PSD proteins are tightly associated with a diverse array of synaptic plasticity, for example long-term synaptic potentiation (LTP) and long-term synaptic depression (LTD), by undergoing assembly and disassembly, clustering and diffusing, and membrane insertion and internalization (Zhu et al., 2016).

PSD-95 is considered as the most important organizer to shape the architecture of PSDs and interface with upstream glutamate receptors and recruit downstream SAPAP-SHANK

complexes (Zhu et al., 2016). By using EM tomography, it is observed in rat hippocampal cultures that the core of PSD is dominated by vertically oriented filaments and by ImmunoGold labeling, PSD-95 is identified as a component of these filaments (Moore et al., 2007). The paralogs of PDS-95 constitute the DLG-MAGUK family of proteins, which includes additionally PSD-93, SAP97 and SAP102 (Kim and Sheng, 2004). According to phylogenetic position and protein domain architecture, the DLG-MAGUK family can be classified into several different groups or subfamilies: calcium/calmodulin-dependent protein kinase (CASK), palmitoylated membrane proteins (MPPs), the Zonula occludens (ZO) proteins, Caspase recruitment domain family (CARMA), Calcium channel β subunit (CACNB), and membrane-associated guanylate kinase with an inverted repeat (MAGI) (de Mendoza et al., 2010).

The signature structure of MAGUK family consists of three PDZ (PSD-95 Discs large Zona occludens 1) domains, a Src homology 3 (SH3) domain, and a catalytically inactive Guanylate kinase-like (Rasband et al.) domain. PDZ domains are modular protein-interaction domains of about 100 amino acids, which are specialized for binding to short peptide motifs at the extreme carboxy terminals (C-term) of other proteins and play an essential role in assembling signaling transduction (Kim and Sheng, 2004). The PDZ domains are normally classified into three types based on the consensus sequence of the last four amino acids of the PDZ-binding motif of their ligands (Harris and Lim, 2001), and all the MAGUK family proteins contain Type I PDZ domains. SH3 domain usually binds proline-rich sequences (PXXP) present on the ligands (Alexandropoulos et al., 1995), but binding partners for MAGUK's SH3 domain have not been identified.

In the human homolog of DLG (hDlg/SAP97), there is an alternative splicing with two proline-rich insertion, I1B, located N-terminal to the PDZ repeats, which makes hDlg capable of binding several SH3 domain-containing proteins and modulate the level of protein oligomerization (McLaughlin et al., 2002). It has been shown that there is a direct binding between the N-terminal segment of SAP97 and the SH3 domain of PSD-95 and this interaction is critical for the synaptic transportation and clustering of AMPA receptors (Cai et al., 2006). The MAGUK GK domain evolved from the yeast GK enzyme and gradually lost its enzymatic activity during evolution, although the critical residues for its binding to phosphate group of GMP are still conserved (te Velthuis et al., 2007). Thus MAGUK GK domain is able to interact with a large amount of proteins from synapses and other cell compartments (Zhu et al., 2016). In addition to the PDZ-SH3-GK common domain architecture, some MAGUKs (PSD-93β, PSD-95β and Sap97β) contain a LIN2-LIN7 (L27) domain, which allows hetero-interactions and homo-

interactions (Parker et al., 2004). By alternative splicing, the DLG gene family can express MAGUKs either with a prototypic N-terminal L27 domain (β-isoforms), or with a putative palmitoylation motif of N-term cysteines (α-isoforms) (Parker et al., 2004; Schlüter et al., 2006). PSD-93 and PSD-95 are expressed mostly as α-isoforms, while the main isoform of SAP97 is β-isoforms, which can homo-multimerize or hetero-multimerize with other L27 domain-containing proteins, such as Lin-2/CASK, (Lee et al., 2002) and Veli (Leonoudakis et al., 2004). The heteromeric L27 interaction is considered to be essential for SAP97 in assembling a number of large protein complexes involved in cell polarity establishment and maintenance (Doerks et al., 2000).

1.3.2 PDZ domain-mediated target interaction

Many synaptic transmembrane receptors, including glutamate receptors (NMDA-type glutamate receptors, NMDARs, and AMPA-type glutamate receptors, AMPARs), synaptic adhesion molecules and ion channels, contain conserved PDZ-binding motifs at their C-terminal cytoplasmic tails (Zhu et al., 2016). The interaction mediated by PDZ domains are crucial in the targeting, clustering, internalizing and trafficking of these synaptic transmembrane proteins. For example, the PDZ1 and PDZ2 domains of PDS-95 can directly interact with the PDZ binding motifs of NR2 subunits of NMDARs, and also recruit AMPARs via indirectly binding with stargazin and other transmembrane AMPAR regulating proteins (TARPs) (Kornau et al., 1995; Chetkovich et al., 2002; Schnell et al., 2002;). These direct and indirect interactions are required for the trafficking, stabilizing and clustering of both AMPARs and NMDARs at synapse during synaptic development and transmission (Chen et al., 2015).

PDZ domain-mediated interaction can associate with the internal peptide segments or phospholipids, but the interaction between MAGUKs PDZ domains and synaptic proteins are through C-terminal peptide motif. Like most domains mediating protein-protein interaction, PDZ domains are relatively small and the primary sequences normally consist of 80-90 amino acids. The peptides fold into a compact globular fold with N- and C- terminals that are close to each other in the structure and compose five to six β-strands (βA-βF) and two α-helices (αA and αB). It has been shown that the PDZ domain-binding motifs of the binding partners associate as an antiparallel β strand in a groove between βB strand and αB helix (Tiwari and Mohanty, 2014). PDZ domains can be divided into three classes (class I-III) based on the sequence of C-terminal motifs of their binding partners. The two most prominent PDZ domain classes are class I and II.

Class I PDZ domains prefer peptides containing S/T-X-Φ-COOH, where a hydrophobic amino acid (Φ) locates at position 0 (C-terminal), followed by any amino acid (X) at position -1, and then Serine or Threonine at position -2. For class II PDZ domains, the typical motif is Φ-X-Φ-COOH (Daqrouq et al., 2015). In DLG-MAGUK family, all three PDZ domains in each member are class I, but they have specific binding partners.

Recent studies suggested an important theme that additional amino acid residues beyond the canonical PDZ domains and their binding motifs often have an impact on the specificity of targeting and the stability of PDZ domain-mediated interaction. PSD-95 can interact with glutamate receptors (NMDARs) and ion channels via the binding of its first two PDZ domains, which are connected with a five-residue but highly conserved linker (Ye and Zhang, 2013). Conjoined PDZ domains are usually connected with short polypeptide linkers so that the interdomain mobility is limited. Furthermore, the interdomain orientation between PDZ1 and PDZ2 of PSD-95 is partially fixed, which thus facilitates synergistic binding to multimeric partners (McCann et al., 2011).

1.3.3 SAP97 alternative splicing

SAP97 is a mammalian MAGUK family protein and widely expressed throughout the brain and other organs, especially in epithelial cells. It has been shown that SAP97 is highly enriched in excitatory synapses, regulates the trafficking and localization of glutamate receptors and thus modulate synaptic plasticity (Fourie et al., 2014). There are two alternatively spliced insertions located between the N-term domain and the first PDZ repeat, termed as I1A and I1B, which can bind SH3 domains and modulate the level of self-association. Additionally, the region between SH3 and GK domains has been characterized as containing four alternatively spliced insertions, I2, I3, I4 and I5, and SAP97 with I1A, I1B and I3 is identified as the most prevalent isoform and been involved in recruiting multiple SH3-containing proteins to the PSD (McLaughlin et al., 2002).

In addition to spliced insertions, it has been identified that the existence of another isoform, α-isoform, in which the prototypic N-term L27 domain of the predominat β-isoform is replaced by double cysteines that are normally palmitoylated (Schlüter et al., 2006). The N-terminal domains confer different subsynaptic localization onto SAP97, and preferentially target SAP97α to PSD and transfer SAP97β to presynaptic and postsynaptic sites or perisynaptically in dendritic spines (Naim et al., 2005; Waites et al., 2009). These different localizations of SAP97 isoforms have

been characterized to reflect their distinct functions in neurons. SAP97α influence AMPA receptor-mediated synaptic strength independent of activity, whereas the effect of SAP97β are regulated by activity in a CaMKII-dependent manner (Schlüter et al., 2006).

1.3.4 SAP97 and binding partners

SAP97 directly associates with NMDARs via the GluN2A/B subunit PDZ-bind motif and the first two PDZ domains of SAP97 (Leonard et al., 1998), and the CaMKII-dependent phosphorylation of SAP97 controls NMDAR trafficking and insertion (Mauceri et al., 2007). In hippocampus, another non-conventional pathway has been characterized, in which SAP97 is involved in the targeting of NMDARs to dendritic spines (Jeyifous et al., 2009). This study revealed that NMDARs were trafficked from somatic endoplasmic reticulum (ER), to a dendritic ER subcompartment, and subsequently to Golgi outposts via a SAP97β- and CASK-dependent manner. When this non-conventional pathway is not available, NMDARs can be transported to PSD via the conventional pathway, which makes the targeting of NMDARs into synapses more efficient and meanwhile enable the local modulation of NMDAR synaptic insertion near PSD (Jeyifous et al., 2009). Further study implied that SAP97 preferentially interacts with GluN2A subunit rather than GluN2B and SAP97 overexpression can facilitate NMDAR subunit switch coupled with larger and faster NMDAR currents in utero, suggesting a potential role of SAP97 in controlling both the number and the specific subunit composition of synaptic NMDARs (Howard et al., 2010).

As a MAGUK family protein, SAP97 can directly interact with many transmembrane AMPAR regulatory proteins, but it is the only member that directly associates with AMPAR subunit GluA1 (Leonard et al.). It is also shown that the N-terminal 104 amino acid residues of SAP97 binds the C-terminal cargo binding domain of myosin VI, a minus-end-directed actin-dependent motor, therefore serve as a molecular link between GluA1 and Myosin VI during the trafficking of AMPARs to the PSD (Ehlers, 2000; Wu et al., 2002). Studies of synaptic consequences of SAP97 overexpression have been inconsistent. Some studies suggested that overexpression of SAP97 leads to an enhancement of the amplitude of AMPR EPSCs in hippocampal slices and the frequency in dissociated hippocampus neuronal cultures (Nakagawa et al., 2004; Rumbaugh et al., 2003; Snyder et al., 2001), while some others observed no effect (Schnell et al., 2002). Furthermore, it has been identified that different isoforms of SAP97 influence AMPAR-mediated synaptic strength in different manners. SAP97α modulates AMPA receptor-

mediated synaptic strength independent of activity, whereas the effect of SAP97β is regulated by activity in a CaMKII-dependent manner (Schlüter et al., 2006). Further studies characterized that both SAP97 isoforms can directly regulate the localization and dynamics of GluA1-containing AMPARs within spines and influence the responsiveness of excitatory synapses to glutamate, but they bind and retain these AMPAR subunits within distinct cell-surface domains (Waites et al., 2009).

Another important binding partners of SAP97 is A-kinase-anchoring protein 79/150, which binds to the regulatory subunit dimer of PKA to direct the holoenzyme to discrete subcellular structure and provides a scaffold for three signaling enzymes, PKA, PKC and calcium-dependent phosphatase calcineurin (Colledge et al., 2000). Furthermore, the SH3 and GK domain of SAP97 can mediate binding to AKAP, and this interaction can enhance the phosphorylation of AMPARs and thus position PKA near the receptor (Colledge et al., 2000).

1.3.5 DLG-MAGUKs and potassium channels

Structural and mutational analyses revealed PSD-95 and other MAGUKs bind to a consensus PDZ-binding sequence (xS/TxV) at the COOH terminus of Kv1 α subunits via either of the first two of the three PDZ domains located near the NH2 terminus (Kim and Sheng, 1996; Tiffany et al., 2000). In most mammalian central neurons, Kv1 channels are predominantly found in the axons, while MAGUKs are found at synapses, most prominently in the postsynaptic density. However, affinity purification assay showed MAGUKs as prominent components of Kv1 complexes from mammalian brain (Schulte et al., 2006). Functional and biochemical evidence suggested that the cell-surface clustering of Kv1 channels are mediated by PSD-95, as a result of direct binding of cytoplasmic tails (COOH terminus) of Kv1 channel α subunits to the PDZ domains of PSD-95 (Kim et al., 1995). Further evidence showed that PSD-95 exhibits robust colocalization with Kv1.1 and Kv1.2 in cerebellar basket cell terminals and the juxtaparanodal regions of nodes of Ranvier, and plays a major role in modulating juxtaparanodal Kv channel activity (Rasband and Trimmer, 2001). In contrast, another study indicated that the clustering of Kv1 channels was normal in mutant mice lacking juxtaparanodal PSD-95, and compensatory over-expression of other MAGUKs did not appear to be involved in maintaining Kv1 channel localization at this site (Rasband et al., 2002).

SAP97 has been reported to play a regulatory role in the localization and trafficking of voltage-gated potassium channels. Early studies demonstrated that SAP97 could bind the C-term of

Kv1 channels via its first two PDZ domains and mediate their clustering (Kim et al., 1995). Biochemical studies showed that SAP97 could directly interact with Kv1.4 and regulate its surface expression (Kim and Sheng, 1996; Tiffany et al., 2000). Similarly, Kv1.5 was also shown to be associated with SAP97. In *Xenopus oocytes*, Kv1.5-encoded outward potassium currents were augmented by the overexpression of SAP97, suggesting SAP97 may play a role in modulating Kv1.5 function (Murata et al., 2001). Another study also characterized the colocalization between SAP97 and Kv1.5 in rat ventricular myocytes and an enhancement of Kv1.5 current in HEK cells co-transfected with SAP97 (Eldstrom et al., 2003). Furthermore, several isoforms from human and rat myocardium have been identified to have distinct effects on the functional properties and membrane expression of Kv1.5 channels (Godreau et al., 2003). A more recent study in cardiac myocytes showed an increased clustering of endogenous Kv1.5 at myocyte-myocyte contacts by using immunocytochemistry when overexpressing SAP97, coupled with an enhancement of both the ultra-rapid activating delayed rectifier potassium current (I_{Kur}) and the number of 4-aminopyridine (4-AP) sensitive potassium channels in cell-attached membrane patches. In live myocytes, SAP97 overexpression induced Kv1.5 organization in plaque-like clusters with poor mobility, suggesting SAP97 could regulate Kv1.5 by retaining and immobilizing Kv1.5 in the plasma membrane (Abi-Char et al., 2008).

In the hippocampus, SAP97 directly bind Kv4.2, which is a key component of the A-type potassium channel, in a conventional PDZ domain-motif binding manner (Gardoni et al., 2007). In this study, it was shown that both SAP97 and Kv4.2 were enriched in PSD fractions and SAP97 regulated the subcellular localization of Kv4.2. Additionally, this regulation depends on the CaMKII-mediated phosphorylation of SAP97 (Ser-39), and the mutation of this site abolished its interaction with Kv4.2 and its modulatory function in governing Kv4.2 membrane distribution (Gardoni et al., 2007). It has been revealed that SAP97 and Kv4.2 are both phosphorylated by CamKII. The CamKII-dependent phosphorylation of Kv4.2 (Ser438 and Ser459) increased surface Kv4.2 expression and the introduction of constitutively active CaMKII resulted in a decrease in neuronal excitability in response to current injections in hippocampal neurons (Varga et al., 2004). The phosphorylation of SAP97 (Ser 232) disrupted the association with GluN2A and the constitutively phosphorylated SAP97 (S232D) decreased GluN2A/SAP97 co-clustering in transfected COS-7 cells (Gardoni et al., 2007). In heart, SAP97 has been shown to interact with the C-term of cardiac Kv4.2 and Kv4.3, which account for a large part of the cardiac transient outward potassium current (I_{to}), and colocalized with Kv4.3 in Chinese Hamster Ovary cells (CHO cells). Similarly with the mechanism in hippocampal neurons,

CaMKII activity is also required for the surface localization of Kv4.2 and its association with SAP97 (El-Haou et al., 2009).

1.3.6 DLG-MAGUKs and GPCRs

In neurons, GPCRs are expressed in the postsynaptic density of dendritic terminals and it is believed that some DLG-MAGUKs provide direct contact with GPCRs (Feng and Zhang, 2009). Early studies have identified that PSD-95 could antagonize the agonist-induced internalization of serotonin 2A receptor (5-HT2AR), which is the main excitatory receptor subtype among the GPCRs for serotonin (Xia et al., 2003). Moreover, it has also been reported that the dissociation of PSD-95 from 5-HT2AR is coupled to recruitment of β-arrestin2 and it may imply that regulatory role of PSD-95 in 5-HT2AR internalization by its competitive binding (Schmid and Bohn, 2010).

SAP97 shares 60% sequence identity with PSD-95, but very less is known about the role of SAP97 in the modulation of GPCR activity. Nevertheless, SAP97 has also been shown to be essential for the phosphorylation of extracellular signal regulated kinase 1/2 (ERK1/2) mediated by corticotropin-releasing factor receptor 1 (CRFR1), which belongs to the GPCR family of proteins and is a target for the treatment of psychiatric diseases such as depression, schizophrenia, anxiety disorder and bipolar disorder (Dunn et al., 2013). Similarly, the 5-HT2AR-stimulated ERK1/2 activation is also regulated by SAP97 (Dunn et al., 2014). However, in contrast to the regulation of adrenergic receptors, the SAP97-mediated modulation of CRFR1 and 5-HT2AR by facilitating ERK1/2 phosphorylation does not require interactions with the PDZ-binding motifs, suggesting that SAP97 may play a global role in regulating GPCR-mediated ERK1/2 activity which is independent of PDZ domain-motif interaction (Dunn and Ferguson, 2015; Dunn et al., 2013; Dunn et al., 2014; Sheng and Sala, 2001).

It has been reported that SAP97 could directly interact with adrenergic receptor family proteins, such as β1-AR, in the conventional PDZ domain-motif binding manner. Proteomic array screening with the C-term of β1-AR suggested that SAP97 is a novel binding partner of β1-AR (Magalhaes et al., 2012) (He et al., 2005). The association between β1-AR and SAP97 and other PDZ-domain containing proteins was later confirmed by biochemical and functional assays (Gardner et al., 2007; Valentine and Haggie, 2011). This association could scaffold PKA to β1-AR, and SAP97 thus facilitated PKA-mediated phosphorylation of Ser312, which is critical for the internalizing and trafficking of β1-AR to membrane, indicating SAP97 is involved in

recycling and resensitization of β1-AR (Gardner et al., 2007). In cardiomyocyte-like cells, the PDZ domain-motif interaction is required for the confinement of β adrenergic receptors, both β1-AR and β2-AR, and C-terminal mutation (to alanines) that obviated the association with PDZ domain-containing proteins increased β-AR diffusion, implicating the interaction contributed to the tethering of β-AR (Valentine and Haggie, 2011).

Another study focusing on characterizing the domains in SAP97 involved in recycling and resensitization of β1-AR by using SAP97 knockdown and rescue strategy showed that PDZ2 domain of SAP97 displayed the highest affinity in binding to β1-AR and regulate its trafficking and functional resensitization, whereas I3 domain contained the binding domains for other proteins, such as AKAP79. The deletion of I3 domain from SAP97 did not affect the binding of SAP97 to β1-AR, but abolish the incorporation of AKAP79/PKA into the SAP97- β1-AR complex (Nooh et al., 2014).

1.4 Scope of the thesis

Extensive physiological and behavioral studies have demonstrated that NE/β2-adrenergic signaling could facilitate the induction of spike-timing-dependent plasticity (STDP) at excitatory synapses (Lin et al., 2003; Seol et al., 2007; Zhou et al., 2013) and enhances memory (Ji et al., 2003; Lemon et al., 2009; Miranda et al., 2011; Tronel et al., 2004), potentially through inhibiting dendritic potassium conductance and therefore increase the propagation of dendritic action potentials (Hoffman and Johnston, 1999; Watanabe et al., 2002). However, it remains unknown which potassium channels are involved in the modulation of dendritic excitability by NE/ β2-adrenergic signaling, and how these potassium channels are mobilized in response to β2-adrenergic receptor activation.

Our previous results suggested that activation of β2-adrenergic receptor by NE treatment facilitated spike-timing-dependent long-term synaptic potentiation at excitatory synapses in hippocampus by inducing dendritic Kv1.1-containing potassium channels inactivation, and this modulatory effect is mediated signaling scaffold SAP97 (data provided by Dr. Yanling Liu). These results narrowed down Kv1.1 as a neuronal substrate to mediate adrenergic modulation in dendritic excitability, and indicated the role of SAP97 in linking adrenergic signaling and potassium channels.

The scope of my work is to study the mechanism of dendritic Kv1.1-potassium channels desensitization, and characterized how SAP97 linking NE/β2-adrenergic signaling to potassium channel inactivation. In this study, I used imaging approaches with cell surface-labeling procedure to characterize the surface expression of Kv1.1-containing potassium channels in response to adrenergic signaling. Since it has been identified SAP97 could interact with the C-terminus PDZ-motifs of Kv1 channels (Kim et al., 1995), I therefore addressed the binding specificities of SAP97/Kv1.1 and SAP97/β2AR and tested the requirement of these interaction in modulating potassium channels inactivation. It is well known that activation of β adrenergic receptors by NE binding causes a rise of the intracellular concentration of the second messenger cAMP, and thus activates PKA (Wang et al., 2004) and results in phosphorylation of Kv1.1 at its C-terminal domain (S446), leading to its rapid translocation into the plasma membrane (Winklhofer et al., 2003). I also sought to find out whether the PKA-mediated phosphorylation of Kv1.1 could be responsible for the mobility of potassium channels in response to adrenergic signaling.

2 Materials and methods

2.1 Mice and genotyping

Mouse lines of conditional SAP97 KO (Zhou et al., 2008) and NEX-Cre (Goebbels et al., 2006) were bred and housed in cages of maximally 5 mice with 12 hours light/dark cycle with controlled temperature and humidity, and were provided with food and water ad libitum. Experimental NEX-Cre animals were generated from heterozygous breeding pairs.

Genomic DNA samples were obtained from small tail pieces (1-2mm) at age P0 before dissociated culture preparation. The tissue was lysed in 150µl PBND lysis buffer (10mM Tris, 50mM KCl, 2.5mM $MgCl_2$, 0.1mg/ml Gelatine, 0.45% (v/v) Nonidet P40 (NP40) and 0.45% Tween 20, pH 8.3) supplemented with Proteinase K (Ambion 200µg/ml) at 55°C under 1400 rpm agitation for 3 hours. Samples were then heated up to 99°C for the purpose of inactivating Proteinase K. Genotypes were determined by PCR (Polymerase Chain Reaction) with primer pairs for SAP97 cKO (primers: fwd 5' CCT CTA CAG AAT CTG AGT TGG CTC; rev: 5' TAA GAA GGA TCA ACT GGC AAA GGT G) and Nex-Cre (primers: fwd 5' CTG CCA GGG ACA TGG CCA GG; rev 5' GCA CAG TCG AGG CTG ATC AGC).

PCR mix	
Sample	2.2µl
10x TNK buffer	2µl
dNTPs (Bioline)	2µl
Primer fwd (50µM)	0.2µl
Primer rev (50µM)	0.2µl
H_2O	15µl
Mango Taq polymerase (Bioline)	0.2µl

PCR program			
Cycle step	Temp.	Time	
Initial denaturation	94°C	5'	
Denaturation	94°C	45''	x35
Annealing	56°C	45''	
Extension	72°C	1'	
Final extension	72°C	10'	
	4°C	Hold	

2.2 Molecular biology

2.2.1 PCR

For the amplification of desired DNA fragment, improved PCR method was employed by using Phusion DNA Polymerase (Thermo Scientific) to ensure high fidelity.

PCR mix	
H_2O	Add to 50µl
5x Phusion HF Buffer	10µl
dNTPs (10mM, Bioline)	1µl
Primer fwd (25µM)	1µl
Primer rev (25µM)	1µl
Template DNA	1ng
DMSO	1.5µl
Phusion DNA Polymerase	0.2µl

PCR program			
Cycle step	Temp.	Time	
Initial denaturation	98°C	30''	
Denaturation	94°C	10''	x10
Annealing	56°C	30''	
Extension	72°C	30'/kb	
Denaturation	94°C	10''	x25
Annealing	56°C	30''	
Extension	72°C	30'/kb	
Final extension	72°C	10'	
	4°C	Hold	

2.2.2 PCR product purification

The PCR product was applied to the QIAquick spin column, and purified by using a bind-wash-elute procedure according to the manufacture's protocol from QIAGEN (Hilden, Germany). DNA was eluted with 30µl of 1:10 diluted Elution Buffer (QIAGEN), and then used for the subsequent steps.

2.2.3 Restriction enzyme digestion

For generating compatible ends on the desired DNA fragments for directional insertion into compatible vectors, restriction digestion was performed by using restriction enzymes (NEB and Thermo Scientific) in their suggested buffer system. The reaction system was incubated at 37°C for 2 hours. The digested DNA fragments were separated using 1% TAE gel, and band with desired size was excised under a UV transillumination system. The DNA was extracted from the gel using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The typical digestion reaction has been shown as below:

Restriction digestion reaction mix	
DNA	1.5µg
10x digestion buffer (Grueneberg et al.)	5µl
Restriction enzyme 1	1µl
Restriction enzyme 2	1µl
H_2O	Add to 50µl

2.2.4 Electrophoresis

Digested DNA fragments were separated by agarose gel electrophoresis. The gels were prepared by dissolving agarose (1%) in TAE buffer (4.84g/l Tris, 1.14ml/l Acetic acid, 50µM EDTA). DNA was pre-mixed with Loading Dye was electrophoresed under constant voltage (140V). To visualize the DNA, Ethidiumbromide (Roth) was added to the agarose. To detect the Ethidiumbromide-stained DNA, the gel was exposed to UV light and the size of DNA was determined by using standard DNA ladder (Phage Lambda DNA StyI digest ready-to-use, GENEON).

2.2.5 Ligation

For the cloning protocol, desired DNA fragments were ligated into different vector backbones using a molar ratio of 3:1. The amount of DNA was estimated by using 1µl of purified PCR or

digestion products on a gel. The mixture was incubated at 16°C for 1 hour for cohesive ends, and overnight for blunt ends. The typical ligation reaction has been shown as below:

Ligation reaction mix	
Vector DNA	50ng
Vector	150nl
10x T4 DNA Ligase Buffer	3µl
Ligase	1µl
H_2O	Add to 30µl

2.2.6 Transformation

1µl of ligation product was transformed into 50µl XL1-Blue *Escherichia coli* (*E. coli*) strain (Stratagene, Santa Clara, CA, USA) by using electroporation (Eppendorf 2510 electroporator) at 1800V. Colonies were cultivated on LB agar plate containing ampicillin (100µg/ml, Carl Roth) at 37°C.

2.2.7 Plasmid preparation

For the amplification of plasmids, single colonies were picked up from the bacteria-transformed LB agar plate and inoculated in either 5ml (for mini-prep) or 100ml (for maxi-prep) of LB medium supplemented with ampicillin (100µg/ml). The culture flask was incubated at 37°C with vigorous shaking overnight. The bacteria were pelleted by centrifugation at 7000xg for 10 min.

For mini-prep, plasmid DNA was extracted from 5ml of overnight culture using Zyppy Plasmid Miniprep Kit (Zyppy) according to the manufacturer's instructions. Briefly, it is based on alkaline lysis method (Birnboim & Doly, 1979) that bypasses bacterial culture centrifugation and resuspension steps common to classical plasmid DNA preparation procedures. 7x Lysis Buffer (Zyppy) was directly added into bacterial culture, followed by neutralization (Neutralization

Buffer, Zyppy) and purification using classical centrifugation method (Endo-Wash Buffer and Zyppy Wash Buffer, Zyppy). DNA was eluted by Zyppy Elution Buffer (Zyppy).

For maxi-prep, we also applied alkaline lysis method. Basically, Buffer P1 (50mM Tris-HCl, 10mM EDTA, 100µg/ml RNase A, pH8.0), Buffer P2 (200mM NaOH, 1% w/v SDS) and Buffer P3 (3.0M Potassium acetate, pH5.5) were added sequentially to lyse bacteria from 100ml overnight culture. Samples were incubated at 55°C for 10 min to catalyze the degradation of RNA. Then the samples were applied on Phase Lock Gel Heavy-tubes (5 PRIME) and mixed with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, AppliChem) to purify the plasmid DNA from all protein contaminations. Phase separation was achieved by centrifugation and the clear aqueous phase was mixed with equal volume of chloroform (Carl Roth). Plasmid DNA was precipitated by adding 0.7x volume of isopropanol (AppliChem) and eluted by Elution Buffer (Qiagen).

2.3 Cell culture

2.3.1 HEK cells transfection

HEK 293 cells (Human embryonic kidney cells) were used for biochemistry assay (Co-immunoprecipitation and GST-pull down assay) and lenti-virus production. HEK293 cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Millipore) supplemented with 10% Fetal bovine serum (FBS, Biochrom). Cells were cultured in 10 cm cell culture dishes at 37°C until they reach a confluence of 70-80% when they were transferred to a 6-well cell culture dish for transfection.

For biochemistry assay, overexpression constructs for Kv1.1, SAP97 or Flag-tagged β2AR were transfected into HEK293 cells using TransIT transfection reagent (Mirus) according to the manufacturer's instructions. Briefly, approximately 2.5µg of plasmid DNA was mixed with 7.5µl of TransIT in Opti-MEM I reduced-Serum Medium (Mirus), and the mixture was applied directly on HEK293 cells after 25 min incubation. Cells were harvested 48 hours after transfection.

For lenti-virus production, plasmid DNA of interest was transfected into HEK293 cells together with HIV-1 packaging vector Δ8.9 and envelope glycoprotein vector vsvg, mediated by TransIT transfection reagent. The cells were incubated at 37°C for 24 hours and moved to 32°C to promote the productivity. Lenti-virus was harvested 48 hours after transfection.

2.3.2 Harvesting and purification of lenti-viral particles

For small-scale lenti-virus production, the supernatant from the transfected dishes was carefully collected and centrifuged at 2000xg for 5 min in order to get rid of the cell debris. The supernatant containing lenti-viral particles was aliqoted and stored at -80°C for further usage.

For large-scale lenti-virus production, the supernatant from all the transfected dishes was pooled and centrifuged at 2000xg for 10 min to isolate lenti-viral particles from the cell debris. The supernatant was then filtered through PVDF membrane with 0.45µm pore size (Millipore), and the lenti-viral particles in the flow-through were pelleted by ultra-centrifugation of 25000 rpm (Beckmann L8-60M with rotor 70Ti) at 4°C for 2 hours. The pellet was dissolved in 100µl virus storage buffer (20mM Tris-HCl, pH8.0), aliqoted and stored at -80°C for further usage.

2.3.3 Dissociated hippocampus neuronal culture

Dissociated neuronal cultures were prepared from hippocampus of P0 mice. Coverslips for neuronal culture were pre-washed overnight in 96% Ethanol, flamed and placed into culture plates, followed by coating with 300µl PDL (Sigma) for 24 hours in 37°C cell culture incubator. Before dissection, coverslips were washed twice with sterile water, dried in laminar flow hood and returned to cell culture incubator after adding 1 ml plating media in each well. The animals were anesthetized on ice for several minutes and decapitated. The brains were carefully removed and kept in dissection solution (mGBSS). Two hemispheres were separated respectively with micro-dissecting tweezers, and meninges were gently removed. Hippocampus was isolated from both hemispheres, transferred to Enzymatic Solution (50mM Na-EDTA, 11.39mM L-cysteine, 10mM $CaCl_2$, 1N HCl, 10 ml DS, 100µl Papain, 10 mg/ml DNase I), put on a mild rotation at 37°C for 30min. After enzymatic digestion, Enzymatic Solution was replaced by Inactivation Solution (25mg BSA, 10mg/ml DNase I in 5% Serum Medium). After removing Inactivation Solution, hippocampus was gently triturated in 5% Serum Medium with fire-polished Pasteur pipette for a couple of times and allowed to settle down for 2 min. The supernatant was transferred to a fresh tube and the undigested chunks were triturated gain in 5% Serum Medium with fire-polished Pasteur pipette. The cell suspension was centrifuged for 5min at 500xg and the pellet was resuspended in 1ml per mouse of 5% Serum Medium. Cells were counted in a Neubauer chamber and 25000 cells were plated into each well. In order to restrict the growth of

astrocytes, FUDR (5-Fluoro-2'-deoxyuridine, Sigma Aldrich) was added on DIV4. Cultures were fed with fresh medium without Glutamate and Aspartate on DIV7.

2.3.4 Virus infection

Small or large-scale lenti-virus carrying plasmid of interest was applied on dissociated hippocampus neuronal cultures on DIV4 or DIV7. 100µl of small-scale or 1µl of large-scale lenti-virus was used to infect neurons in each well of 24-well culture plates.

2.3.5 Immunofluorescence labeling of cell surface Kv1.1

In order to detect the presence of a protein in neurons, it is common to decorate cells with antibodies following cell permeabilization, which could disrupt the cell membrane sufficiently to allow the passage of antibodies. The effect of disruption is exacerbated at the level of plasma membrane, which is the cellular membrane most exposed to detergents used for cell permeabilization. To study the localization, surface stability and endocytosis of ion channels (Kv1.1) in hippocampal dissociated neuronal cultures, we immune-labeled the protein on the surface with a specific antibody against an extracellular epitope. Without permeabilization antibody could not have the access through plasma membrane, and therefore only proteins remaining on the cell surface could be decorated by extracellular antibody. Using this method, we established that Kv1.1 was internalized from the plasma membrane after Norepinephrine treatment.

Briefly, low-density hippocampal neuronal cultures from floxed SAP97 mice (DIV17) were treated with Norepinephrine (10mM in Artificial cerebrospinal fluid (ACSF, 119mM NaCl, 26mM $NaHCO_3$, 20mM Glucose, 2.5mM KCl, 1mM Na_2HPO_4, 1.3mM $MgSO_4$, 2.5mM $CaCl_2$)) at 37°C for 10 min to induce the internalization of potassium channels (Kv1.1). Coverslips as required were washed once with Phosphate-buffered saline (PBS, w/o Ca^{2+}, w/o Mg^{2+}, Millipore) supplemented with NBQX (2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo[f]chinoxalin-2,3-dion, 10µM) and then removed with tweezers to another dish with freshly prepared ICC Fixation Solution (4% paraformaldehyde and 4% Sucrose in PBS w/o Ca^{2+}, w/o Mg^{2+}, pH6.9-7.2) at room temperature for 8 min. If too much background fluorescence was present when imaging, Quenching Solution (25mM Glycine) for eliminating free aldehyde groups was applied on coverslips. Coverslips with fixative was rinsed with PBS (w/o Ca^{2+}, w/o Mg^{2+}), followed by 30

min of incubation at room temperature in Goat Serum Dilution Buffer (GSDB, 450mM NaCl, 2% Goat serum in 20mM phosphate buffer). Surface Immunocytochemistry for endocytosis assay does not require permeabilization since detergent destroys membranes. For intracellular antigens detection, 0.3% Triton X-100 was added into GSDB (with 2% Goat serum) for permeabilization. Primary antibodies were diluted in GSDB (with 2% Goat serum) and applied on coverslips at 4°C overnight. Coverslips were gently washed in GSDB (w/o goat serum) three times for 10 minutes each, followed by one time with PBS (w/o Ca^{2+}, w/o Mg^{2+}) for 30 minutes. Secondary antibodies were also diluted in GSDB (w/o goat serum) and applied on coverslips for 2 hours at room temperature in a dark environment. Double labeling experiments (2 different antibodies from 2 different species) are best carried out by sequentially incubating with the primary and secondary antibodies. Coverslips were washed three times with GSDB (w/o goat serum) three times for 10 minutes each, one time with 5mM Sodium Phosphate Buffer for 15 minutes. When all the necessary washing steps have been completed, the coverslips can be counter stained with DAPI or Hoechst (10 µg/ml) to stain the nuclei. Coverslips were then mounted onto glass slips with 8µl of MOWIOL-DABCO Mounting Medium (0.1M Tris, 2.5% Glycerol, 10% MOWIOL and 2.5% DABCO), and stored at -20°C protected from light.

2.3.6 Image analysis and quantification

Images were acquired using a Carl Zeiss LSM710 confocal microscope. Images for all conditions in a particular experiment were obtained using identical acquisition parameters, and unadjusted whole images were analyzed using NIH ImageJ software, applying identical analysis parameters. Each experimental condition was represented by at least six coverslips from more than three different cultures, ~8-12 images were acquired per coverslips, and three dendrites were analyzed per neuron. Quantification of surface Kv1.1 was performed by defining the outlines of neuronal dendrites with NIH ImageJ and calculating the Kv1.1 fluorescence in this area. These values were then normalized to the average surface fluorescence of untreated control cells.

2.4 Biochemistry

2.4.1 Sample preparation

For analysis of dissociated hippocampus neuronal cultures, neurons on DIV17 were washed with ice-cold PBS (w Ca^{2+}, w Mg^{2+}), scraped out by cell scraper (Sarstedt), and harvested in 2x sample buffer (3.4% Tris, 3.3% Tris-HCl, 0.02% Sucrose, 0.03% EDTA, 8% SDS, 0.02% bromophenol blue, 0.27% DTT w/v). 40µl of 2x sample buffer was used for each well of 24-well dishes. Samples were heated up to 80°C for 5 min and stored at -80°C for further usage.

2.4.2 Co-immunoprecipitation

In order to study protein interaction between Kv1.1, SAP97 and β2AR, we performed Co-Immunoprecipitation (CoIP), which is one of the most widely used methods for capturing and purifying the primary target as well as other macromolecules bound to the target. Principally, overexpression constructs for Kv1.1, SAP97 and Flag-tagged β2AR were co-transfected into HEK293 cells mediated by TransIT (Mirus). 48 hours after transfection, the growth medium was removed from the cell, followed by twice of washing with PBS (w/o Ca2+, w/o Mg2+). Cells were incubated at 4°C for 30 min after adding ice-cold FLAG IP Lysis buffer (50mM Tris-HCl, 150 mM NaCl, 1mM EDTA, 0.5% Triton X-100, pH7.5) supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche Life Science). Cells were then scraped and collected into a fresh pre-cold EP tube followed by centrifugation at 12,000xg for 10 min. The supernatant was transferred to a fresh tube used for CoIP assay. Protein G-agarose beads (Roche Applied Science) were washed beads twice with PBS and restore to 50% slurry with PBS. Cell lysate was pre-cleared by adding 40µl of Protein G-agarose and incubated at 4°C for 1 hour with gentle agitation (using a rotating wheel). After pre-clearing, agarose beads were pelleted by centrifugation (20 s at 5,000 x g, 4°C) and the supernatant was removed to a fresh pre-chilled EP tube. 2µg of antibody was directly added into the sample followed by 2 hours incubation at 4°C with gentle agitation (using a rotating wheel). The samples were then transferred to 50 µL of homogeneous protein G-agarose suspension (25 µL bed volume), and incubated overnight at 4 °C with gentle agitation (using a rotating wheel). Agarose-antibody-antigen complexes were collected by centrifugation (20 s at 5,000 x g, 4°C) and rinsed twice with FLAG IP Lysis buffer, twice with Washing Buffer II (50mM Tris-HCl, 500mM NaCl, 0.5%Triton X-100, pH7.5), and once with twice with Washing Buffer III (50mM Tris-HCl, 0.5% Triton X-100, pH7.5). The pellet was

resuspended in 50µl of 2x Sample Buffer, and proteins were denatured by heating the suspension to 80°C for 3 minutes and analyzed by SDS-PAGE.

2.4.3 GST Pull-Down assay

To identify and characterize the interaction between SAP97 PDZ domains and the C-terminus PDZ binding motifs of Kv1.1 and β2AR, we performed GST Pull-Down assay, which is a typical experiment to identify interactions between a probe protein and targets. The coding sequence of probe protein, GST-fused SAP97 N-terminus, was cloned into an Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible expression vector. The expression vector was introduced into electroporation-competent cells (SURE, XL1-Blue, DH5α) by electroporation (1800V). Colonies were cultivated on LB agar plate containing ampicillin (100µg/ml, Carl Roth) at 37°C, and inoculated into a 5ml overnight culture at 37 °C using a shaker for uniform growth. The bacterial culture was Dilute cultures 1:50 in 2x4 mL LB and incubate at 37 °C and 4µl of 1M IPTG was added into each culture after 3 hours. After another 1.5-2 hours of incubation, bacteria cells were pelleted by centrifugation at 4000 rpm for 15 minutes at 4 °C and drained thoroughly on ice. Bacterial pellets (from 4 mL of culture) were resuspended by 200µl of GST Pull-down Lysis Buffer (20mM Tris-HCl, 200mM NaCl, 1mM EDTA, 0.5%NP-40, pH8.0) supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche Life Science), sonicated in an ice water bath (10x30s, H) and centrifuged at 16000xg for 15 minutes at 4 °C. The supernatant was transferred to a pre-chilled fresh EP. The sepharose 4B was washed twice with PBS (w/o Ca2+ and Mg2+) and one time with GST Pull-down Lysis Buffer. Sufficient sepharose 4B beads slurry (40 µL for each reaction/2x4 mL culture) was added into bacterial cells lysate and incubated at room temperature for 1 hour followed by three time of washing with PBS (w/o Ca2+ and Mg2+) and one time with GST Pull-down Lysis Buffer.

Overexpression constructs for Kv1.1 and β2AR were transfected into HEK293 cells mediated by TransIT (Mirus), and proteins were extracted as described before (same as protein extraction for Co-immunoprecipitation). HEK293 cell lysate was added to GST-beads, and incubated at 4 °C overnight. The beads were washed for five times with HEK293 cell lysis buffer, and boiled for 5 min following adding 40µl of 2x sample buffer. The samples were analyzed by SDS-PAGE.

2.4.4 BCA assay

The concentration of protein from hippocampus or transfected HEK cells was determined by applying BCA quantitation assay by using BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. For each batch of experiment, quantitation was accompanied with a standard curve measurement, containing 9 different concentration of BSA diluted from the original standard BSA sample.

2.4.5 SDS PAGE and Western blotting

For the biochemical analysis of protein samples extracted from brains, dissociated hippocampus neuronal cultures and transfected HEK293 cells, proteins were applied on a denaturing SDS-PAGE (Shapiro et al., 1967) in 10% Bis-Tris gel at 100V for 3 hours, followed by wet western blotting (Towbin et al., 1979). The gels for SDS-PAGE were prepared following the give recipe:

10% Resolving Gel	
AMPA	6.7ml
3.5x Bis-Tris Buffer	6.0ml
10% SDS	100µl
10% APS	120µl
TEMED	25µl
H_2O	7.3ml

3.5% Stacking Gel	
AMPA	1ml
3.5x Bis-Tris Buffer	0.8ml
10% SDS	100µl
10% APS	96µl
TEMED	20µl
H_2O	6.2ml

After electrophoresis, protein was transferred onto a 0.2µm nitrocellulose membranes membrane (GE Healthcare) in transfer buffer (25mM Tris, 191mM Glycin, 20% Methanol) at 200V at 4°C for 2 hours. Ponceau S dye (Sigma Aldrich), a non-specific protein dye, was used to confirm a successful protein transferring. The membrane was next blocked with 5% milk in Tris-Buffered Saline-Tween 20 (TBS-T, 20mM Tris-HCl, 140mM NaCl, 0.1% Tween 20, pH7.6) at room temperature for 30 min, and incubated with primary antibody (information was shown as below) diluted in 5% milk in TBS-T at room temperature for 2 hours or at 4°C overnight with mild

shaking. The membrane was washed 3 times with TBS-T and incubated with secondary antibody at room temperature for 2 hours in a dark environment. The membrane was then washed 3 times with TBS-T and once with TBS (TBS-T, 20mM Tris-HCl, 140mM NaCl, pH7.6). The membrane was scanned using Odyssey Infrared Imaging System (Li-COR Bioscience). Proteins were scanned using 700nm or 800nm channels and analyzed by Odyssey analysis software using integrated intensity with background correction.

List of primary antibody used for immunoblotting

Antibody	Host	Dilution	Source
Primary antibody			
Kv1.1	Mouse	1:1000	NeuroMab
SAP97	Rabbit	1:2000	Abcam
β2-AR	Rabbit	1:500	Santa Cruz
Flag	Mouse	1:2000	Sigma Aldrich
Mortalin	Mouse	1:5000	Neuromab
GFP	Mouse	1:2000	Neuromab
Primary antibody			
IRDye 800CW	Goat	1:10000	Odyssey Licor
IRDye 680CW	Goat	1:10000	Odyssey Licor

2.5 Data analysis

Data from repeated runs of the same experiments was pooled and Student t-test followed by one-way ANOVA was used for statistical analysis of the difference between groups. The significance in the data was indicated as: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

For data from immunocytochemistry experiments, the sample size (n) is indicated by n = number of coverslips. Each experimental condition was represented by at least six coverslips from more than three different cultures, ~8-12 images were acquired per coverslips, and three dendrites were analyzed per neuron. Quantification of surface Kv1.1 was performed by defining the outlines of neuronal dendrites with NIH ImageJ and calculating the Kv1.1 fluorescence in this area. These values were then normalized to the average surface fluorescence of untreated control cells.

3 Results

3.1 NE induces reduction of Kv1.1 surface expression

The expression of K^+ channels is extremely diverse due to multiple gene families, heteromultimeric combination of α and β subunits, splicing variants and post-translation regulation (Guan et al., 2006). It has been shown that Kv1.1 could be locally synthesized in dendrites, and Kv1.1 local translation could be regulated by NMDA-dependent synaptic activity (Raab-Graham et al., 2006). Another study characterized the dynamic regulation of dendritic expression of Kv1.1 in hippocampus during associative learning.

Our preliminary work found that the activation of adrenergic receptors by Norepinephrine (NE) treatment in hippocampus CA1 pyramidal neurons promoted spike timing-dependent (STD) long-term synaptic potentiation by inactivating dendritic Kv1.1-containing potassium channels. A plausible mechanism would be the NE-induced reduction in surface expression of Kv1.1.

In order to test this hypothesis, we cultured low-density hippocampal neuronal cultures from floxed SAP97 mice and measured the surface expression of Kv1.1 by a cell surface labeling procedure. In this procedure, we performed immunocytochemistry with an antibody against an extracellular epitope, which could detect the surface expression of Kv1.1 in non-permeabilized neurons (Fig 3.1A). The specificity of Kv1.1 surface labeling was confirmed by two experiments. First, a Kv1.1 antibody against the intracellular cytoplasmic tail did not decorate non-permeabilized neurons, but after permeabilization, the antibody accessed the intracellular antigenic epitopes and decorated neurons (Fig 3.1A). Second, Kv1.1 immuno-reactivity was almost undetectable with the Kv1.1 antibody against the extracellular epitope in neurons, in which Kv1.1 was knocked-down by a short hairpin RNA (shKv1.1) by RNA interference (Fig 3.1B). Together, these results indicate that the detection of Kv1.1 immuno-reactivity by this cell surface procedure with an antibody against the extracellular epitope was specific for Kv1.1 surface signaling.

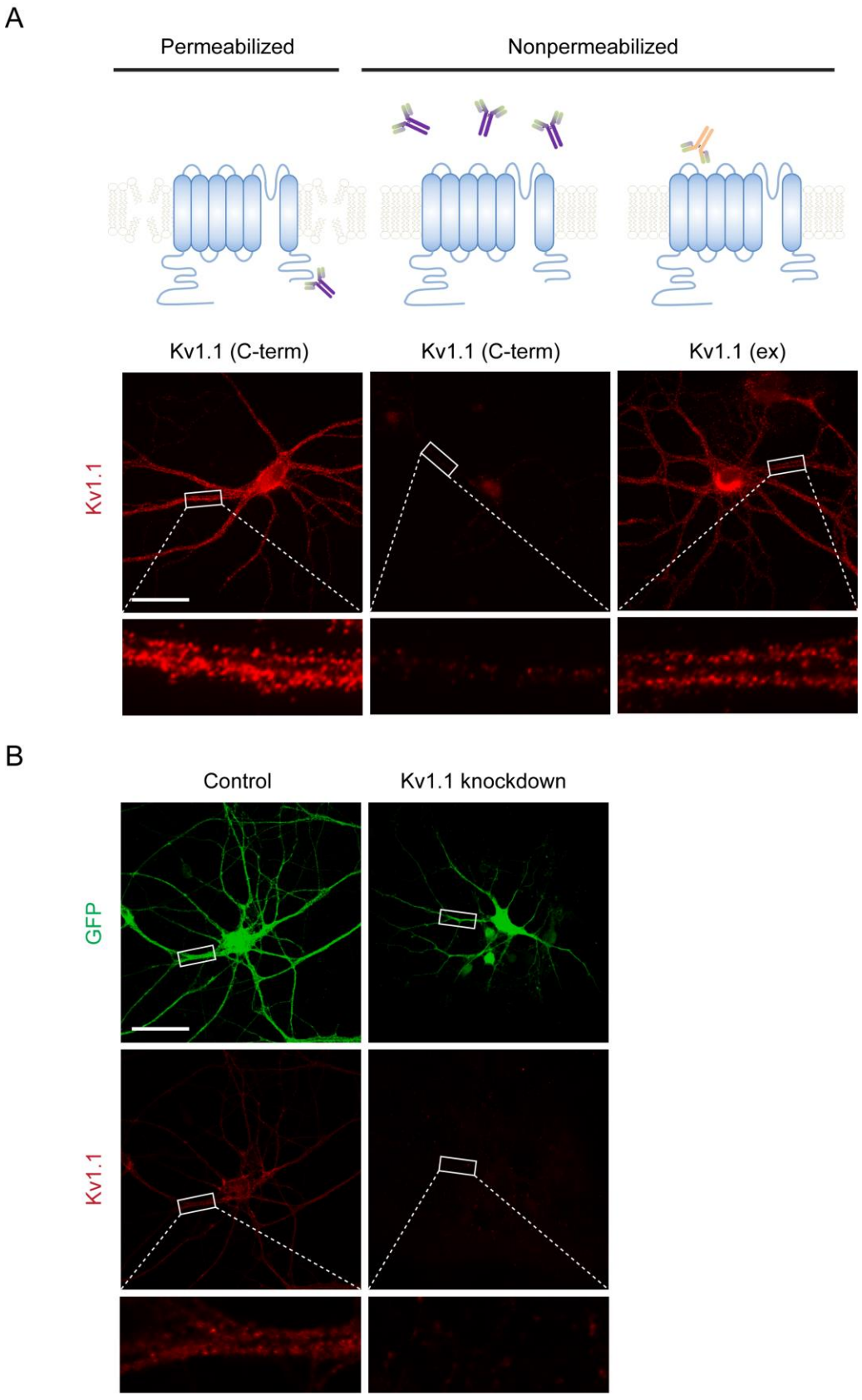

A
Permeabilized
Nonpermeabilized
Kv1.1 (C-term)
Kv1.1 (C-term)
Kv1.1 (ex)
Kv1.1
B
Control
Kv1.1 knockdown
GFP
Kv1.1

Figure 3.1 Antibody against an extracellular epitope decorates surface Kv1.1 by using a cell surface labeling procedure.

(A) Specificity of either permeabilized or non-permeabilized immuno-staining conditions was tested together with different Kv1.1 antibodies. Low-density hippocampal neuronal culture was prepared from floxed SAP97 mice, and DIV17 neurons were used for immunocytochemistry. An antibody directed against the intracellular cytoplasmic tail failed to label Kv1.1 in non-permeabilized neurons, while decorating neurons after permeabilization. However, the antibody against an extracellular epitope decorated neurons without permeabilization**.** **(B)** Neurons expressing shKv1.1, identified by GFP fluorescence, were devoid of immunogenic surface signaling for the Kv1.1 extracellular antibody.

By using a Kv1.1 extracellular antibody and the cell surface labeling procedure, we were able to explore the mechanisms underlying norepinephrine-mediated inactivation of Kv1.1. Hippocampal neurons were treated with 20 µM norepinephrine for 10 min, followed by fixation with 4% Paraformaldehyde and cell surface immuno-labeling with the Kv1.1 extracellular antibody. We quantified the fluorescence intensity of an Alexa Fluor 555-conjugated secondary antibody from secondary dendrites on a confocal microscope. We found that NE treatment induced ~30% reduction of the surface Kv1.1 signal compared to that of control neurons (control, n=12: 100 ± 2.93% vs. NE, n=12: 67.50 ± 3.11%, p<0.0001; Fig. 2A, B).

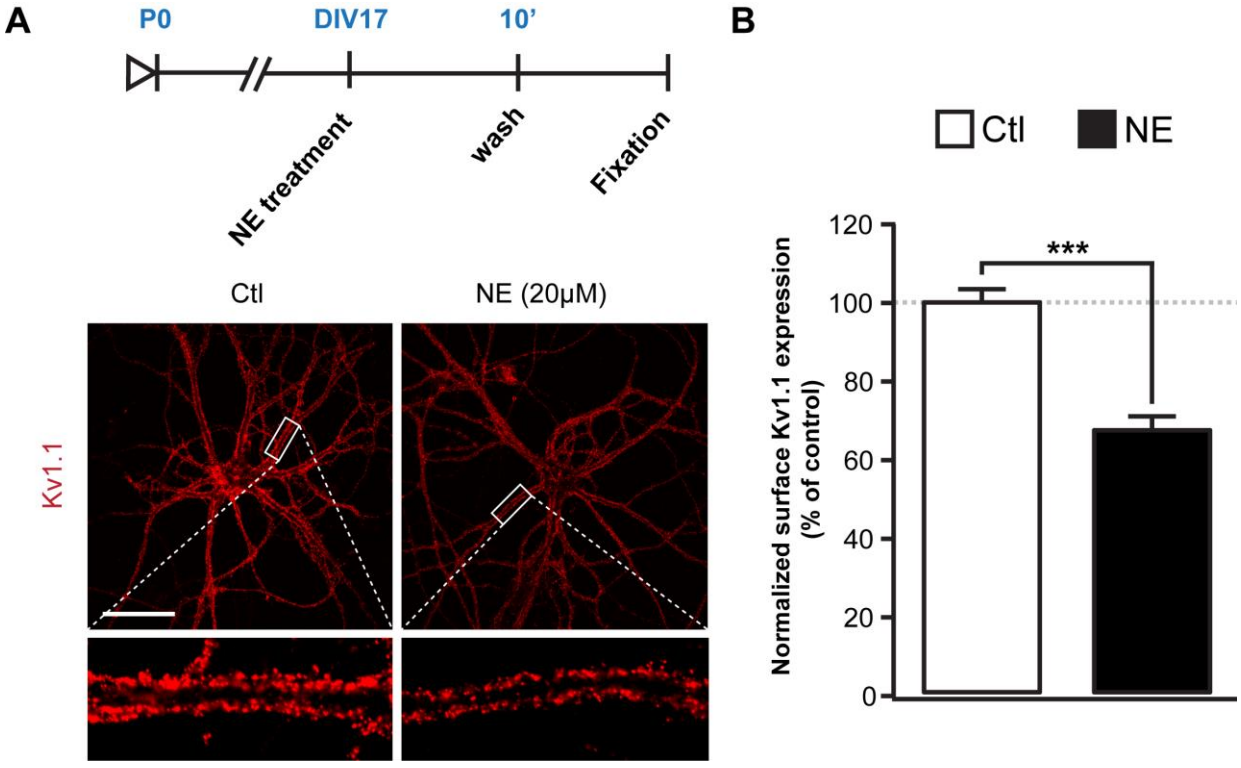
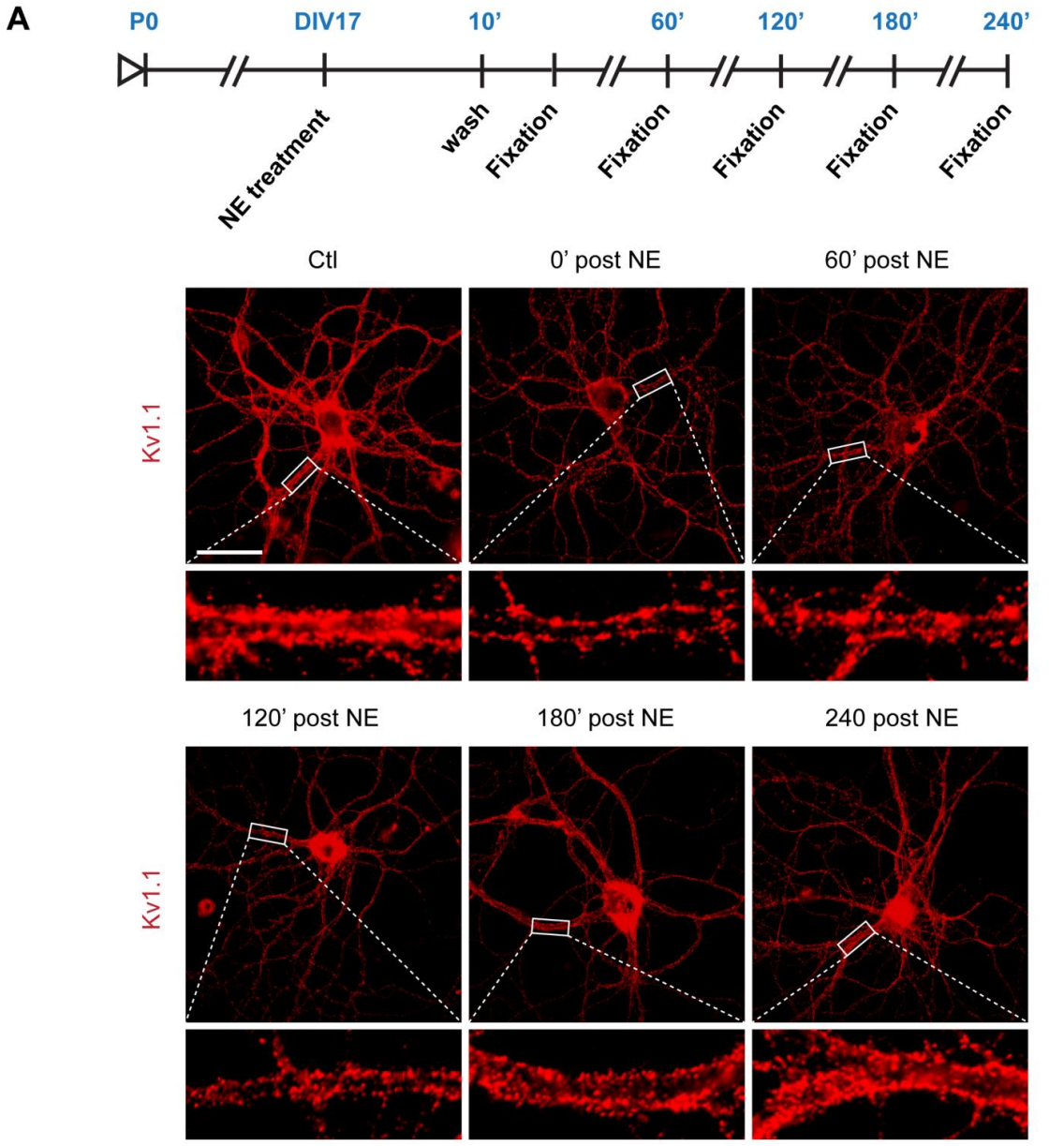


Figure 3.2 NE induced a reduction in Kv1.1 surface expression.

(A) Upper panel illustrates the time line of the primary hippocampal neuron culture with the cell surface labeling procedure. Cultures were treated with 20 µM NE for 10 min and labeled with the Kv1.1 extracellular antibody in the cell surface labeling procedure. The lower panels present a magnified view of a dendritic region, which was analyzed. **(B)** The summary graph of the surface labeling procedure is illustrated. The surface fluorescence was measured relative to that of control sister cultures under the same antibody incubation and image acquisition conditions. Compared to control neurons (left), NE treated neurons showed a reduced level (69.54%) of Kv1.1 surface expression. Statistical significant difference is indicated with *** ($p<0.0001$).

To verify that the absence of surface Kv1.1 after NE treatment was a result of internalization, we analyzed the possible receptor recycling to the plasma membrane after removing the agonist (NE) by washing. We then kept the neurons in the absence of NE for 60, 120, 180 or 240 minutes and fixed them for the cell surface labeling assay. After 10 minutes of NE application (20 µM), ~30% of surface Kv1.1 was removed from the plasma membrane (control, n=6: 100 ± 2.51% vs. 0' post NE, n=8: 57.78 ± 4.50%, $p<0.0001$; Fig. 3.3). When the agonist was washed out, the recycling of Kv1.1 occurred after 60 minutes (60' post NE, n=8: 64.50 ± 4.07%, $p<0.0001$; Fig 3.3 A). The surface expression of Kv1.1 was progressively increased over time until the surface expression level was similar to that of non-treated control neurons after 240 minutes (120' post NE, n=7: 78.21 ± 1.68%, $p<0.0001$; 180' post NE, n=8: 84.45 ± 4.02%, $p<0.001$; 240' post NE, n=6: 103.64 ± 6.84%, $p=0.64$; Fig 3.3 B). These results indicated that NE could induce a reversible internalization of Kv1.1 in hippocampal neurons.


A
P0
DIV17
10'
60'
120'
180'
240'
NE treatment
wash
Fixation
Fixation
Fixation
Fixation
Fixation
Ctl
0' post NE
60' post NE
Kv1.1
120' post NE
180' post NE
240 post NE
Kv1.1

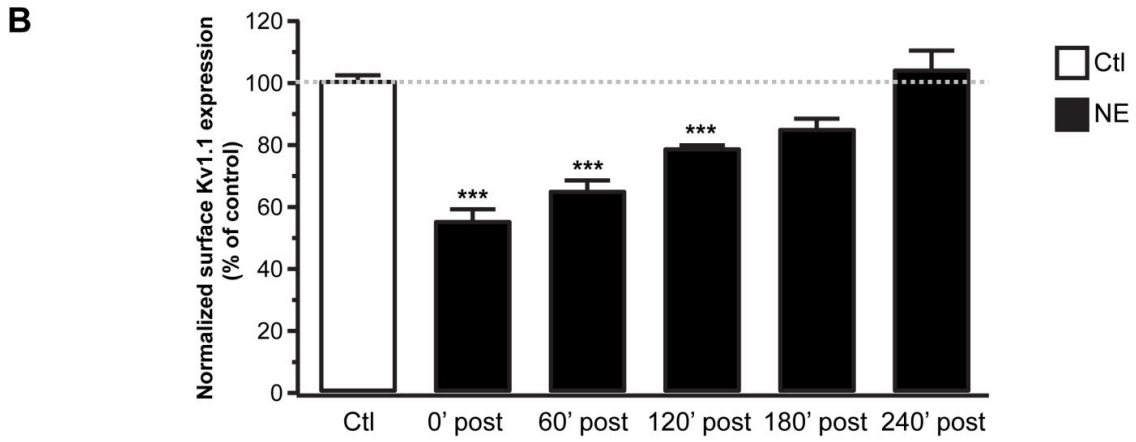

B
Normalized surface Kv1.1 expression (% of control)
120
100
80
60
40
20
0
***
***
***
Ctl
NE
Ctl
0' post
60' post
120' post
180' post
240' post

Figure 3.3 Kv1.1 recycles back to the dendritic plasma membrane after NE removal.
(A) Neurons were incubated in the presence of NE for 45 min at a final concentration of 20 μM, washed with neuronal feeding medium, and then kept in the absence of NE for 60, 120, 180, 240 minutes. They were then fixed and immune-labeled by Kv1.1 extra antibody in the cell surface labeling procedure. **(B)** Summary graph of surface Kv1.1 expression in neurons with different duration of NE wash period (after an exposure to 20 μM for 10 min) in panel A, normalized to untreated control neurons.

3.2 NE-induced Kv1.1 internalization requires β2-adrenergic receptor activity

NE signaling, as a global regulator of the internal state of the brain, still has some selectivity in the activation of various adrenergic receptors in different neurons to permit more specific sculpting of responses in the brain (O'Donnell et al., 2012). In mammalian brain, hippocampus receives noradrenergic innervation and hippocampal neurons express β-adrenergic receptors, which has been implicated to play important roles in modulating the gating mechanism of long-lasting changes in synaptic strength of synaptic plasticity (Gelinas and Nguyen, 2005). Forms of long-term potentiation (LTP) are considered to be critical for long-term storage of spatial and contextual memories in the hippocampus (Review by O'Dell et al., 2015).

To characterize whether β-adrenergic receptors are involved in mediating NE-triggered Kv1.1 internalization, we applied NE on hippocampal neurons pretreated with different β-adrenergic receptor antagonists, and then analyzed the internalization of Kv1.1. We found that the NE-triggered surface Kv1.1 reduction could be prevented in the presence of the preferential β2-adrenergic receptor antagonist ICI118551 (erythro-dl-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol, 1μM for 1h) (control vs. NE+ICI 118551, n=7; 90.43 ± 4.01%, p=0.07; Fig. 3.4A, B), but not by a β1-adrenergic receptor antagonist (1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl) phenoxy]-2-propanol methanesulfonate, 1μM for 1h) (control vs. NE+CGP20712, n=8; 59.05 ± 3.51%, p<0.0001; Fig. 3.4A, B). These results suggested that NE-mediated Kv1.1 internalization is signaled through β2-adrenergic receptor.

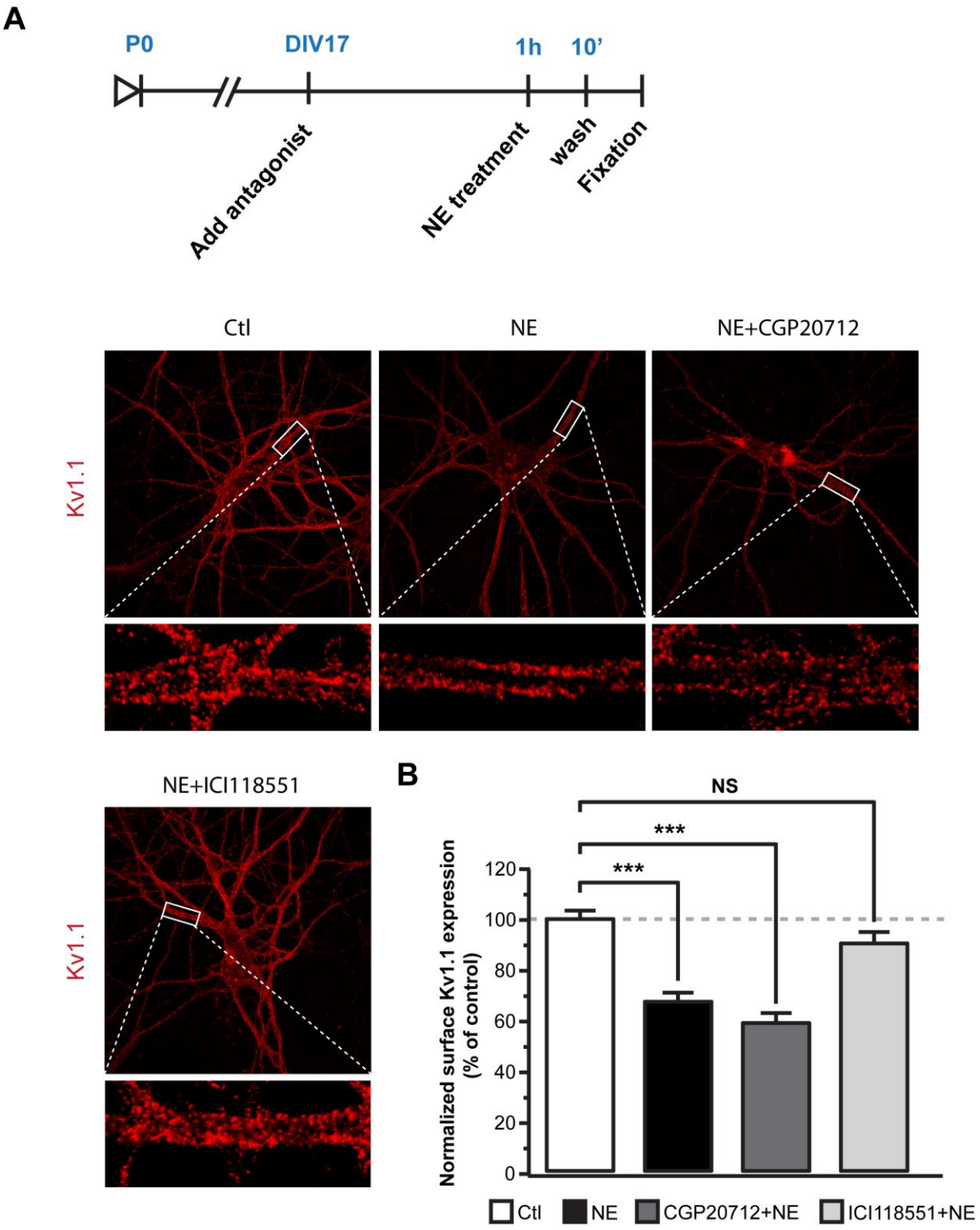

A
P0
DIV17
1h
10'
Add antagonist
NE treatment
wash
Fixation
Ctl
NE
NE+CGP20712
Kv1.1
NE+ICI118551
Kv1.1
B
NS
***
***
Normalized surface Kv1.1 expression (% of control)
120
100
80
60
40
20
0
Ctl
NE
CGP20712+NE
ICI118551+NE

Figure 3.4 NE-mediated Kv1.1 internalization requires β2-adrenergic receptor activity.
(A) Hippocampal neurons were pretreated with β1-adrenergic receptors antagonist CGP20712 (1 μM, 1h) and β2-adrenergic receptors antagonist ICI118551 (1 μM, 1h), followed by NE treatment (20 μM, 10 min) and cell surface labeling with Kv1.1 extracellular antibody. **(B)** Summary graph of surface Kv1.1 expression in neurons treated with different pharmacological reagents in panel A, normalized to untreated control neurons.

3.3 Kv1.1 C-terminal PDZ domain binding ligand is required for the interaction with SAP97

The C-terminus of Kv1.1 with the sequence "KSKLLTDV" contains a class I PDZ domain binding motif (S/T-X-Φ), which binds to class I PDZ domain-containing proteins, such as membrane associated guanylate kinase (MAGUK) family proteins. SAP97, a member of MAGUK family scaffold proteins, has been implied to interact with many voltage-gated potassium channels and G-protein coupled receptors, and plays a regulatory role in their trafficking and localization in neurons. Biochemical studies have revealed a direct binding of SAP97 to the C-terminal PDZ domain binding motifs of several Kv1 family channels, such as Kv1.4 and Kv1.5.

To test whether Kv1.1 and β2-adrenergic receptors interact with SAP97, we performed co-immunoprecipitation assays using the lysate of HEK293 cells transfected with Kv1.1, Flag-tagged β2-adrenergic receptor and SAP97. Kv1.1 antibody immunoprecipitation resulted in co-precipitation of SAP97 and Flag-tagged β2-adrenergic receptors (Fig 3.5A). Conversely, β2-adrenergic receptor precipitation with anti-Flag antibody resulted in co-precipitation of SAP97 and Kv1.1 (Fig 3.5A). These results suggested that Kv1.1 could interact and form a complex with SAP97 and β2-adrenergic receptors.

To test whether Kv1.1 and SAP97 interact in vivo, we precipitated Kv1.1 from hippocampal extracts. The result indicated that SAP97 was co-immunoprecipitated with Kv1.1 in hippocampus (Fig 3.5B). We used conditional SAP97 KO mice, which were crossed with a Nex-Cre driver line to silence the expression of SAP97 in glutamatergic neurons (Goebbels et al., 2006). The remaining immunereactivity may be derived from hippocampal interneurons and astrocytes, which also express SAP97. Most importantly, weaker intensity of SAP97 band in the immunoprecipitate from SAP97 cKO mice compared to wild type mice indicated that Kv1.1 and SAP97 interacted preferentially in glutamatergic neurons. However, β2-AR was not co-immunoprecipitated with Kv1.1.

To determine whether the interaction between Kv1.1 and SAP97 is mediated by the C-terminus of Kv1.1, we transfected SAP97 together with expression constructs of full-length wild-type Kv1.1 or two deletion-mutant Kv1.1, lacking the last three (TDV) or four (LTDV) amino acids into HEK293 cells. We found the full-length wild-type Kv1.1 co-immunoprecipitated SAP97, but the deletion-mutant constructs did not, consistent with previous results, demonstrating an interaction between the Kv1.1 C-terminal PDZ-binding motif and DLG-MAGUKS (Kim et al., 1995). Together, these results implicated that Kv1.1 could bind to SAP97 in hippocampus through its C-terminal PDZ-binding motif.

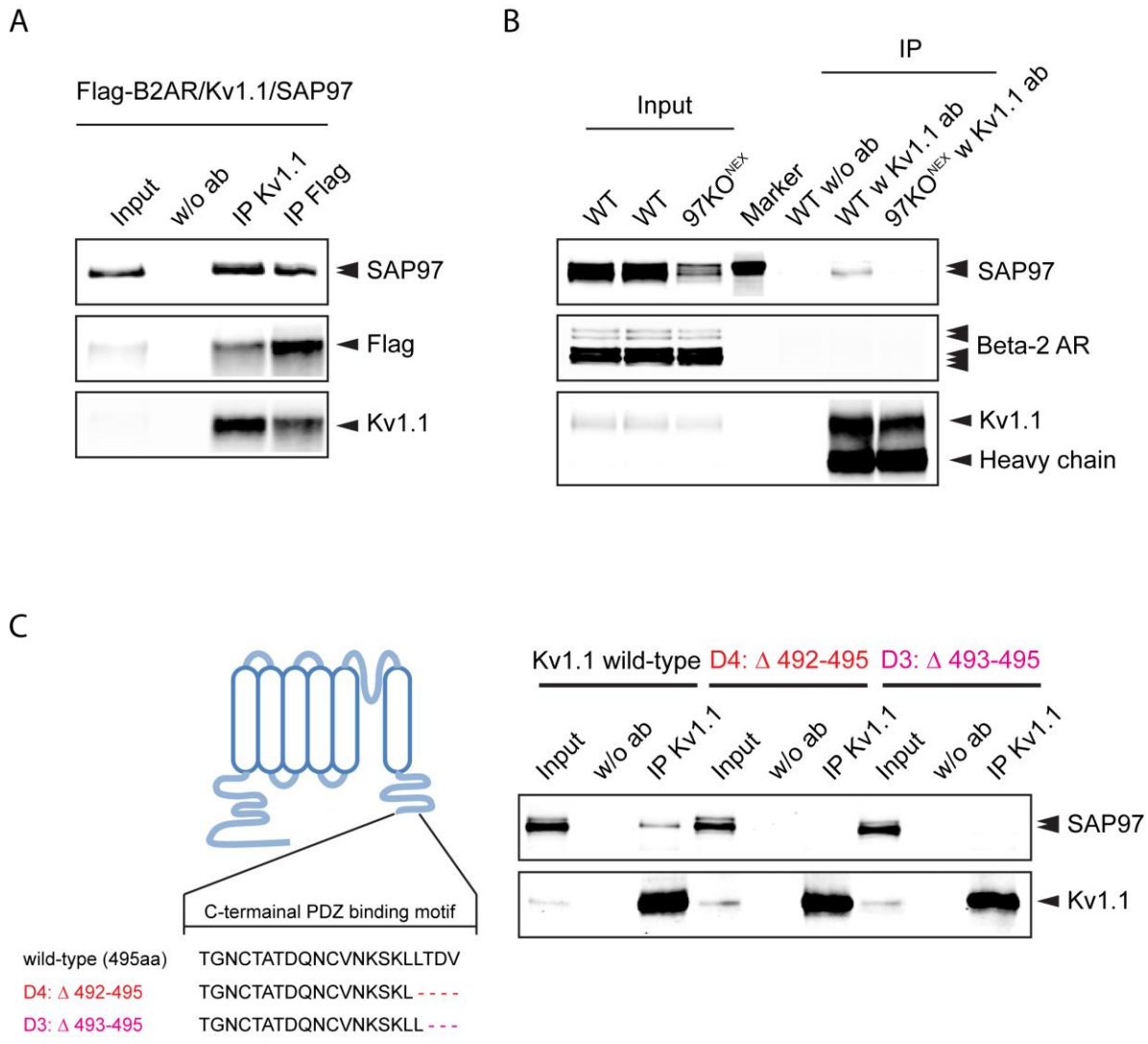


Figure 3.5 Kv1.1 C-terminal PDZ ligand is required for Kv1.1 and SAP97 interaction.

(A) Co-immunoprecipitation of Kv1.1, β2-AR and SAP97 complex. Expression constructs of Kv1.1, SAP97 and Flag tagged β2-AR were co-transfected into HEK293 cells. The lysate was subjected to co-

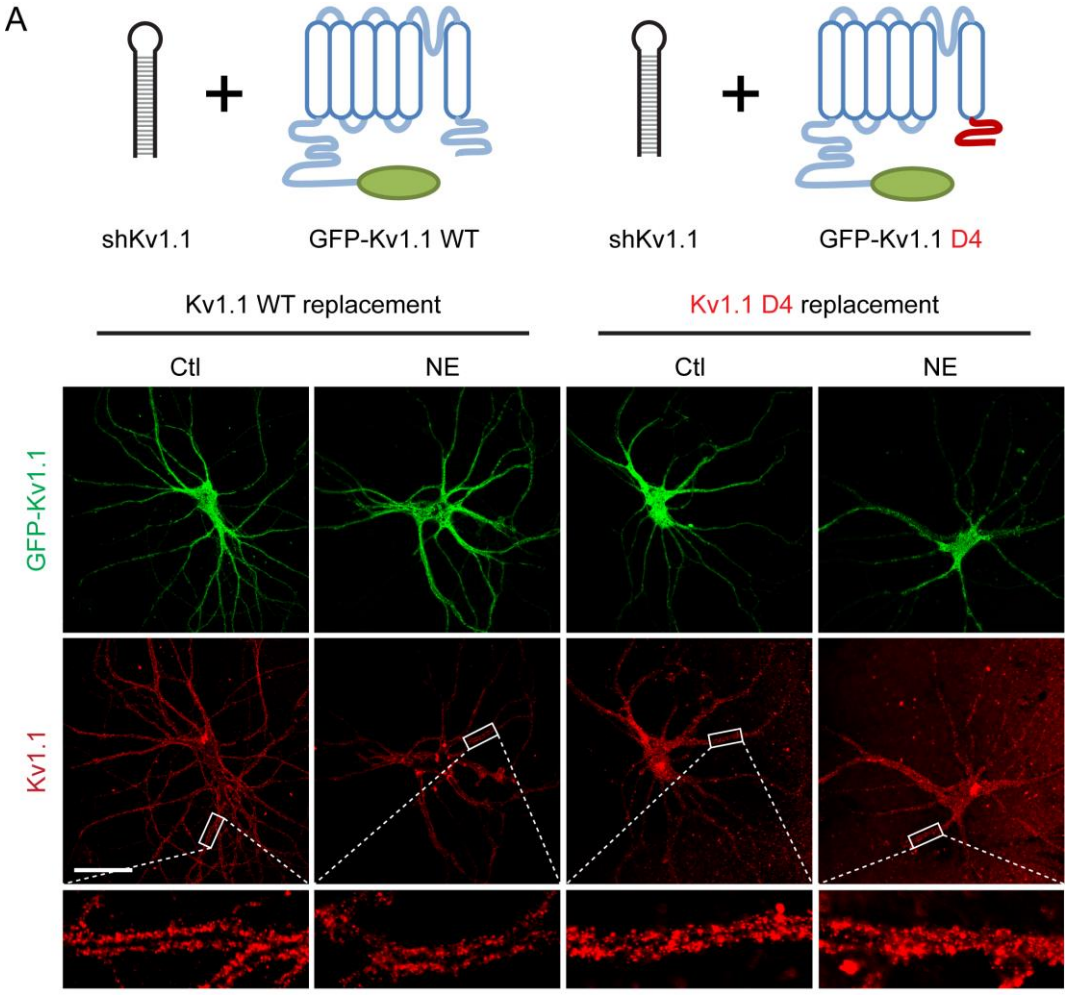
immunoprecipitation with anti-Flag antibody and probed with anti-Kv1.1 and anti-SAP97 antibodies or co-immunoprecipitation with anti-Kv1.1 antibody and probed with anti-Flag and anti SAP97 antibodies. **(B)** Kv1.1 was co-immunoprecipitated from mouse hippocampus from WT mice and SAP97-NEX (97KO^{NEX}) conditional knock-out mice, which lacked SAP97 proteins in forebrain glutamatergic neurons. The extracts were probed with anti- β2-AR and anti-SAP97 antibodies. Lane "WT w/o ab" presents co-immunoprecipitation assay from wild-type mice without primary antibody as control experiment. Lanes "Input" present the hippocampal extracts used for co-immunoprecipitation assay. **(C)** Kv1.1 and SAP97 was co-immunoprecipitated from HEK293 cell extracts, transfected with expression constructs of SAP97 and wild-type Kv1.1, or two mutant Kv1.1 which lack the C-terminal three (D3) or four (D4) amino acids. Lane "w/o ab" presents co-immunoprecipitation assay from HEK293 cell lysates without primary antibody as control experiment. Lanes "Input" present the transfected HEK293 cell lysates used for co-immunoprecipitation assay.

3.4 NE-induced Kv1.1 internalization requires Kv1.1 C-terminal PDZ domain binding ligand

The PDZ domain-mediated interaction between SAP97 and Kv1 channels have been reported to be involved in the regulation of Kv1 channels trafficking and surface localization. Early studies implicated that the binding of SAP97 to Kv1.4 caused an intracellular accumulation of Kv1.4 (Tiffany et al., 2000). In contrast, another study suggested that the interaction of SAP97 with Kv1.5 in cardiac myocytes could retain and immobilize Kv1.5 on the plasma membrane, resulting in more functional channels (Abi-Char et al., 2008).

To test whether the C-terminal PDZ domain binding motif of Kv1.1 is required for its internalization triggered by NE, I applied a "molecular replacement" strategy (Schlüter et al.) by using an shRNA targeting Kv1.1, which knocked-down Kv1.1 by more than 90% in hippocampal cultures when expressed from an adeno-associated viral (AAV) vector (data provided by Dr. Yanling Liu). In order to replace the endogenous Kv1.1, I cloned a bicistronic AAV vector with shKv1.1 and the Kv1.1 cDNA, which had silent mutations in the targeting sequence of shKv1.1. In the second construct, I inserted a Kv1.1 cDNA, lacking the sequence coding the last four amino acids (LTDV) of the C-terminal PDZ domain binding ligand (Fig. 3.6A). Hippocampal neurons expressing the GFP-tagged wild-type Kv1.1 exhibited normal NE-induced internalization (Kv1.1 wild-type replacement control, n=7: 100 ± 3.28% vs. Kv1.1 wild-type replacement NE, n=7: 76.46 ± 2.75%, $p<0.0001$, Fig. 3.6B, C). When Kv1.1 D4 was introduced into neurons to replace the endogenous protein, the surface expression of GFP-tagged mutant

Kv1.1 (Kv1.1 D4) was normal, but punctate structures with Kv1.1 immunereactivity could be detected outside the cells (Fig. 3.6A, B). In contrast to wild-type Kv1.1 replacement, the internalization was impaired in neurons expressing mutant Kv1.1-D4 (Kv1.1 D4 replacement control, n=8: 100 ± 5.45% vs. Kv1.1 D4 replacement NE, n=7: 104.81 ± 8.37%, p=0.62, Fig. 3.6B, C). These results suggested that the PDZ binding motif at Kv1.1 C-terminal was not required for the membrane localization of Kv1.1, but rather for the regulation of Kv1.1 internalization by NE/β2-AR signaling.


A
shKv1.1
GFP-Kv1.1 WT
shKv1.1
GFP-Kv1.1 D4
Kv1.1 WT replacement
Kv1.1 D4 replacement
Ctl
NE
Ctl
NE
GFP-Kv1.1
Kv1.1

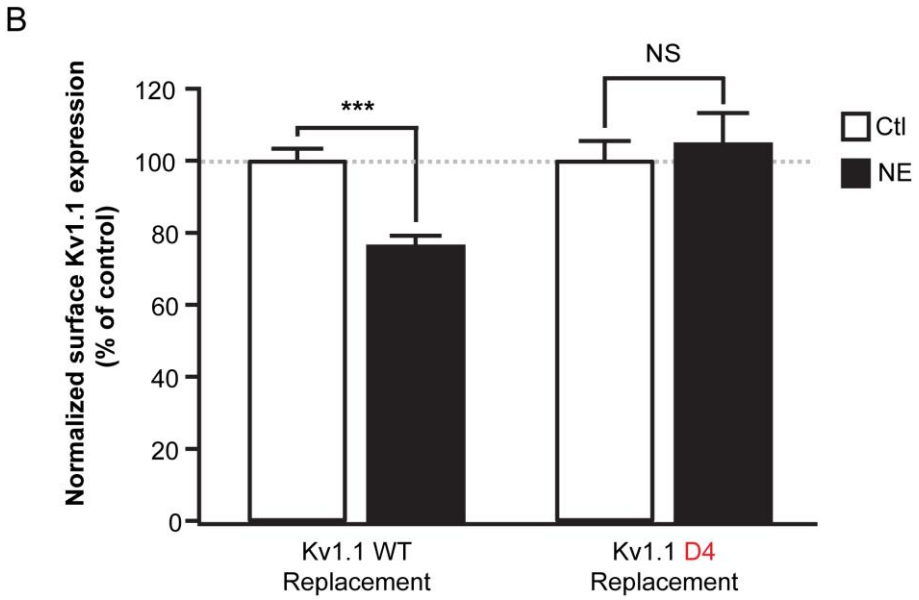

B
Normalized surface Kv1.1 expression (% of control)
120
100
80
60
40
20
0
***
NS
Ctl
NE
Kv1.1 WT Replacement
Kv1.1 D4 Replacement

Figure 3.6 PDZ binding motif of Kv1.1 C-terminal was required for NE-induced Kv1.1 internalization.

(A) Molecular replacement assay of Kv1.1 C-terminal PDZ binding motif. Silent mutations were introduced in the targeting sequence of shKv1.1 into either full-length Kv1.1 or a Kv1.1 deletion construct, which lacked the last four amino acids (LTDV). GFP-tagged full-length Kv1.1 or GFP-tagged Kv1.1 D4 mutation was cloned into bicistronic AAV vector together with highly efficient shRNA to knock-down endogenous Kv1.1. We applied the molecular replacement strategy with these constructs on hippocampal neurons and tested NE-induced Kv1.1 internalization of Kv1.1. **(B)** Summary graph of NE-induced surface Kv1.1 expression reduction in neurons infected with different AAV-mediated Kv1.1 replacements in panel A, normalized to untreated neurons infected with the same replacement virus.

3.5 SAP97 is required for Kv1.1 and SAP97 interaction

I then tested the requirement of SAP97 for NE-induced internalization of Kv1.1. I prepared hippocampal neuronal cultures from floxed SAP97 mice, and knocked-out SAP97 from pyramidal neurons with an AAV on DIV4, expressing a bicistronic expression cassette with GFP and Cre. The neurons were then tested by the NE-mediated internalization assay and cell surface labeling. SAP97 conditional knock-out neurons were identified by their green fluorescence and imaged for fluorescence intensity analysis. The fluorescent signal was normalized to signals from untreated SAP97 conditional knock-out neurons. I found that conditional knockout of Kv1.1 had no effect on the surface expression of Kv1.1in hippocampal neurons. Furthermore, incubation with NE did not change the fluorescence intensity of surface Kv1.1 expression in SAP97 conditional knock-out neurons (SAP97 cKO control, n=6: 100±2.69% vs. SAP97 cKO NE, n=6: 105.07±2.37%, p=0.20, Fig. 3.7A left panel, C). Thus, the removal of Kv1.1 from dendritic surface after NE treatment was abolished in AAV-mediated SAP97 conditional knockout neurons.

To further prove the requirement of SAP97 in the regulation of NE-triggered Kv1.1 internalization, we crossed floxed SAP97 mouse with a NEX-Cre driver line, which knock-out SAP97 expression from principal glutamatergic neurons (Goebbels et al., 2006). I prepared hippocampal neuronal cultures from NEX-Cre-mediated SAP97 knock-out mice ($SAP97KO^{NEX}$), and applied NE-treated internalization assay. Consistent with results from AAV-mediated SAP97 conditional knock-out neurons, NEX-Cre-mediated SAP97 knock-out neurons also showed a normal surface expression of Kv1.1 but an impairment of NE-triggered Kv1.1 internalization ($SAP97KO^{NEX}$ control, n=8: 100 ± 3.04% vs. $SAP97KO^{NEX}$ NE, n=10: 111.57 ±

3.53%, p=0.11, Fig. 3.7A right panel, C). Together these results reveal that SAP97 was required for NE-mediated Kv1.1 inactivation.

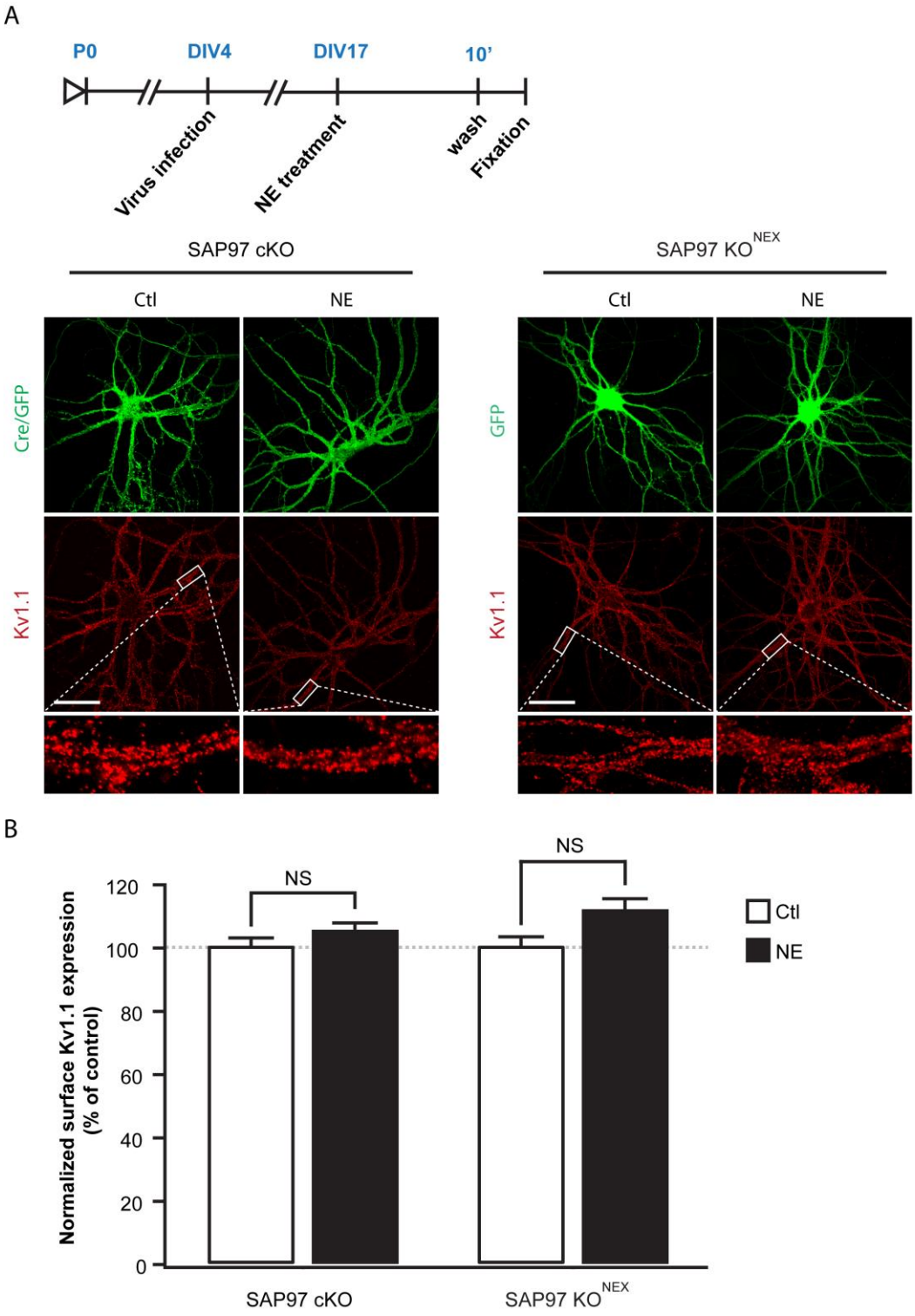


Figure 3.7 NE-induced Kv1.1 internalization was impaired in SAP97 knock-out neurons.
(A) Analysis of AAV-Cre transduced cultures of floxed SAP97 knock-out mice and AAV-GFP transduced cultures of SAP97 KO^{NEX} mice. I prepared hippocampal neuronal cultures from floxed SAP97 mice, and knock-out SAP97 from pyramidal neurons with a bicistronic expression cassette with GFP and Cre. SAP97 cKO neurons were applied with NE for Kv1.1 internalization assay (Left panel, SAP97 cKO). I also crossed the floxed SAP97 with a NEX-Cre driver line, deleting SAP97 expression in principal glutamatergic neurons. Hippocampal neuronal cultures were prepared from P0 SAP97 KO^{NEX} mice and used for NE-induced Kv1.1 internalization assay (right panel, SAP97 KO^{NEX}). (B) Summary graph of surface Kv1.1 expression after NE treatment in AAV-Cre-mediated SAP97 conditional knockout neurons and NEX-Cre-mediated SAP97 knock-out neurons in panel A, normalized to untreated SAP97 knock-out neurons from the same culture.

3.6 PSD-93b rescues the impairment of NE-induced Kv1.1 internalization in SAP97 knock-out neurons

SAP97 and its paralogs constitute the DLG-MAGUK family of proteins, which includes additionally PSD-93, PSD-95 and SAP102 (Kim and Sheng, 2004). All of the proteins in this family share a common structure of five protein-protein interaction domains: three N-terminal PDZ domains (PDZ1, PDZ2 and PDZ3) are followed by an SH3 domain and an enzymatically inactive GK domain. PSD-93, PSD-95 and SAP102 are also known to be expressed in the hippocampus, so the similar structures of different DLG-MAGUKs may provide clues as to whether family members play similar roles in the modulation of hippocampal neuronal activities.

To test whether other DLG-MAGUKs could substitute the role of SAP97 in hippocampal neurons, I examined the effects of overexpressing PSD-93, PSD-95 and SAP102 in SAP97 knock-out neurons. As the most abundant isoform of SAP97 in hippocampus is the β isoform, which contains an N-terminal L27 domain, capable of homo- and hetero-multimerization with itself or other L27 domain-containing protein, such as CASK (Nakagawa et al., 2004), I used the β isoform of the DLG-MAGUK constructs for the experiments. First, I found the loss of NE-triggered internalization of Kv1.1 in NEX-Cre-mediated SAP97 knock-out neurons was rescued by the expression of a GFP-fused SAP97β (SAP97 KO^{NEX} NE, n=12: 107.68 ± 4.76% vs. SAP97 KO^{NEX}+GFP-SAP97 NE, n=10: 79.14 ± 7.31%, $p<0.01$, Fig. 3.8A, B). Second, only the overexpression of PSD-93β reiterated the effect of SAP97 in regulating Kv1.1 internalization after NE treatment (SAP97 KO^{NEX} NE, n=12: 107.68 ± 4.76% vs. SAP97 KO^{NEX}+PSD-93-GFP NE, n=12: 79.30 ± 4.25%, $p<0.001$, Fig. 3.8A, B), but PSD-95beta and SAP102, which does not

contain a beta isoform, did not (SAP97 KO^{NEX} NE, n=12: 107.68 ± 4.76% vs. SAP97 KO^{NEX}+PSD-95-GFP NE, n=12: 102.92 ± 7.76%, p=0.61, Fig. 3.8A, B; SAP97 KO^{NEX}+SAP102-GFP NE, n=9: 96.14 ± 5.94%, p=0.15, Fig. 3.8A, B).

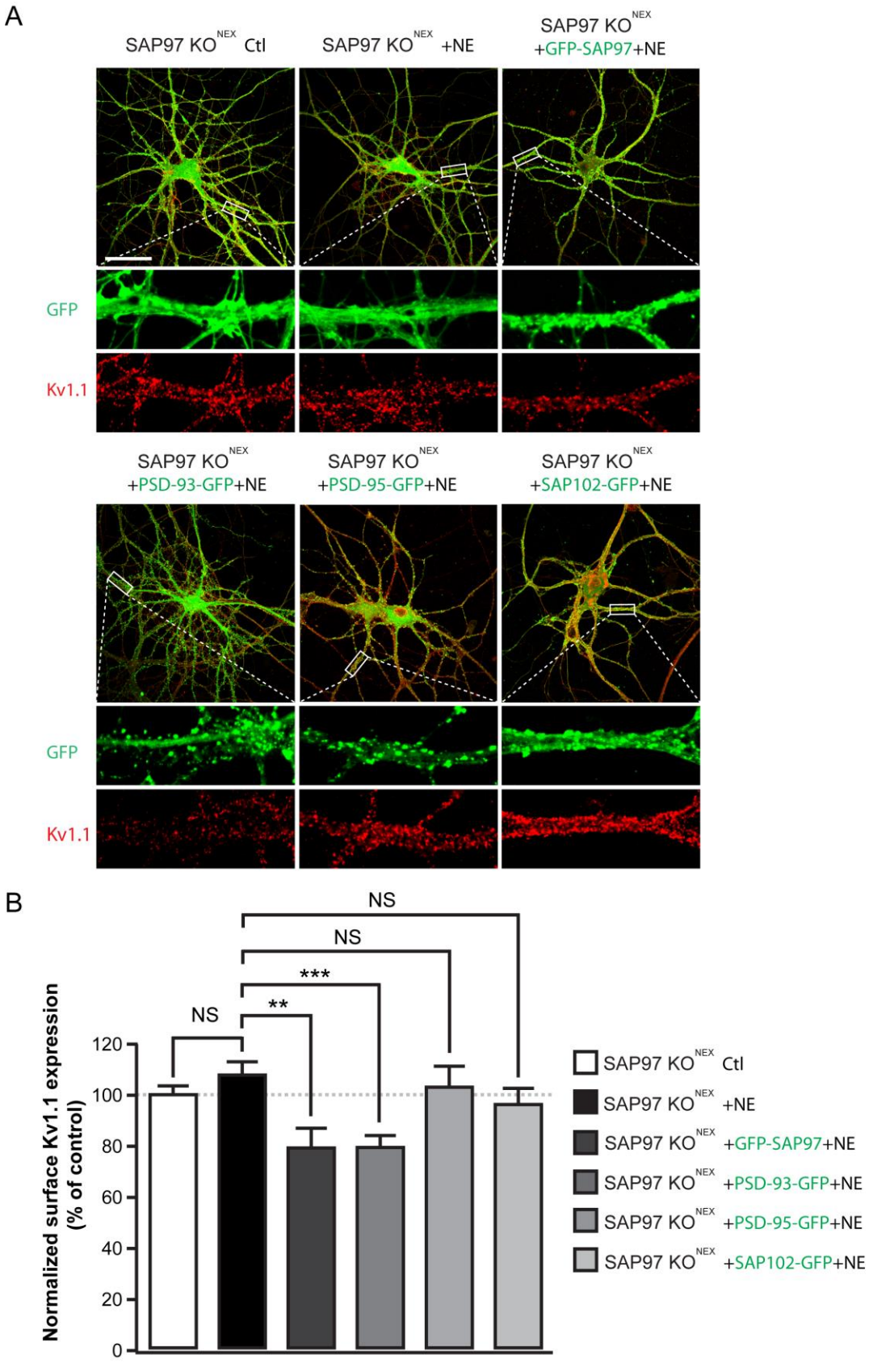

A
SAP97 KO NEX Ctl
SAP97 KO NEX +NE
SAP97 KO NEX +GFP-SAP97+NE
GFP
Kv1.1
SAP97 KO NEX +PSD-93-GFP+NE
SAP97 KO NEX +PSD-95-GFP+NE
SAP97 KO NEX +SAP102-GFP+NE
GFP
Kv1.1
B
NS
NS
***
**
NS
Normalized surface Kv1.1 expression (% of control)
120
100
80
60
40
20
0
SAP97 KO NEX Ctl
SAP97 KO NEX +NE
SAP97 KO NEX +GFP-SAP97+NE
SAP97 KO NEX +PSD-93-GFP+NE
SAP97 KO NEX +PSD-95-GFP+NE
SAP97 KO NEX +SAP102-GFP+NE

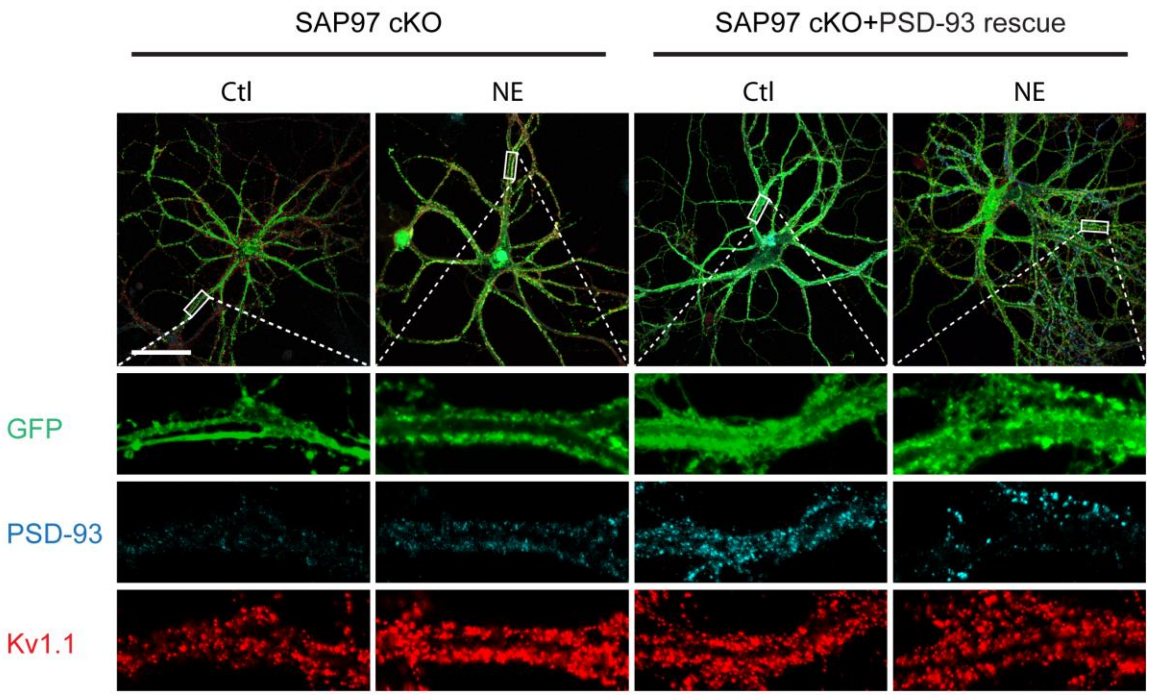
Figure 3.8 SAP97 and PSD-93 overexpression rescue impairment of Kv1.1 internalization in SAP97 knock-out neurons.

(A) Effects of β isoform DLG-MAGUKs overexpression in SAP97 KO^{NEX} neurons. Hippocampal neuronal cultures were prepared from P0 SAP97 KO^{NEX} mice, and lentivirus-mediated overexpression of GFP-fused DLG-MAGUKs was transduced on DIV4. Expression of GFP-SAP97β and PSD-93β rescued the loss of NE-induced Kv1.1 internalization in SAP97 KO^{NEX} neurons, but expression of PSD-95β and SAP102 produced no effect. (B) Summary graph of surface Kv1.1 expression after NE treatment in DLG-MAGUKs overexpressed SAP97 KO^{NEX} neurons in panel A, normalized to untreated SAP97 KO^{NEX} neurons from the same culture.

In order to time the onset of SAP97 knock-out, we prepared hippocampal neuronal cultures from floxed SAP97 mice, and knocked-out SAP97 from pyramidal neurons with AAV-Cre/GFP on DIV4 and overexpressed PSD-93 with lentivirus on DIV7. The neurons were first treated with 20 μM NE for 10 minutes, followed by cell surface labeling procedure to label surface Kv1.1 (Alexa Fluor 555-conjustated secondary antibody). Then I permeabilized the neurons and incubate the coverslips with anti-PSD-93 antibody and another secondary antibody (Alexa Fluor 647-conjustated secondary antibody). SAP97 conditional knock-out neurons with PSD-93 overexpression were identified by their green fluorescence (AAV-Cre/GFP) and enhanced level of PSD-93 signal. I found that the impairment of NE-induced Kv1.1 internalization is partly rescued by overexpression of PSD-93 in AAV-Cre-mediated SAP97 conditional knockout neurons (SAP97 cKO+PSD-93 rescue Ctl, n=8: 100 ± 5.04% vs. SAP97 cKO+PSD-93 rescue NE, n=7: 85.68 ± 4.78%, $p<0.005$, Fig. 3.9A, B).

Together, these results confirmed the crucial role of SAP97 in regulating NE-induced Kv1.1 internalization and indicated that the overexpression of PSD-93 could partly rescue the function of endogenous SAP97 in SAP97 conditional knock-out neurons. Notably, the PSD-95 beta isoform was unable to rescue, indicating a functional difference between the DLG-MAGUK beta isoforms.

A


SAP97 cKO
SAP97 cKO+PSD-93 rescue
Ctl
NE
Ctl
NE
GFP
PSD-93
Kv1.1

B

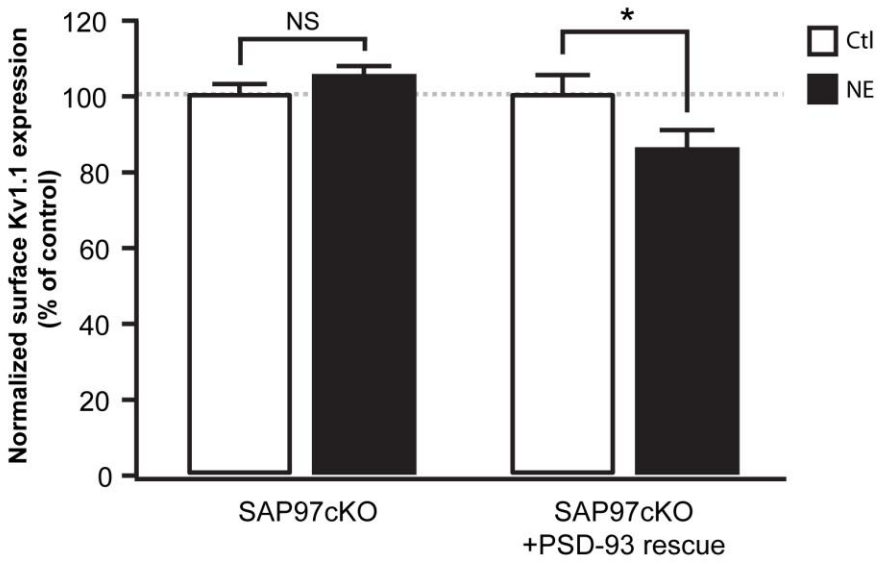

Normalized surface Kv1.1 expression (% of control)
120
100
80
60
40
20
0
NS
*
Ctl
NE
SAP97cKO
SAP97cKO +PSD-93 rescue

Figure 3.9 PSD-93 overexpression rescues impairment of Kv1.1 internalization in AAV-Cre-mediated SAP97 conditional knock-out neurons.

(A) Hippocampal neuronal cultures were prepared from P0 SAP97 floxed mice. AAV-Cre-mediated SAP97 conditional knock-out was transduced on DIV4 and lentivirus-mediated overexpression of PSD-93 was transduced on DIV7. Overexpression of PSD-93 could rescue the impairment of NE-induced internalization in 97 conditional knock-out neurons. (B) Summary graph of surface Kv1.1 expression after NE treatment in PSD-93 overexpressed SAP97 conditional knock-out neurons in panel A, normalized to untreated SAP97 conditional knock-out neurons from the same culture

3.7 SAP97 PDZ12 tandem binds to Kv1.1 and β2AR

DLG-MAGUKs all contain three PDZ domains at their N-terminus, which have been reported to bind a large number of PSD proteins, including NMDA receptors, K+ channels, GPCRs, nitric oxide synthase and cytoskeletal protein cysteine-rich PDZ binding protein (CRIPT) (Brenman et al., 1996; Kim et al., 1995; Kornau et al., 1995; Niethammer et al., 1998). Studies have shown that the inter-domain (linking sequence) between PDZ1 and PDZ2 (together referred to as PDZ12 tandem) is short (five residues) but highly conserved, indicating a molecular basis for synergistic binding with dimeric or multimeric membrane proteins, including NMDA receptors and K+ channels (Feng and Zhang, 2009). This synergistic interaction not only increases the binding affinity between DLG-MAGUKs and the binding partners, but also provides exquisite specificity for the interaction. Early study implied that the PDZ12 tandem of PSD-95 could bind the C-terminus of Kv1.4 and regulate its clustering in heterologous cells (Long et al., 2003). A plausible molecular mechanism for SAP97 in regulating Kv1.1 surface expression could be through direct binding of the PDZ12 tandem.

To test this hypothesis, I made point-mutations in both PDZ1 and PDZ2 domains to abolish their interactions with the C-terminal PDZ domain-binding motifs of Kv1.1 and β2AR. PDZ domains can be divided into three classes (class I-III), based on their sequences, and class I and II are the most prominent PDZ domain classes. Conserved histidine residues in the DLG-MAGUKs PDZ1 and PDZ2 domains, which belong to class I, are predicted to form hydrogen bonds with the threonine at the -2 position in the C-terminus of Kv1.1 and β2AR, as is typical of most class I PDZ domain-ligand interactions. In contrast, class II PDZ domains contain a valine at this position and bind ligands with large hydrophobic residues, such as phenylalanine (Songyang et al., 1997). Thus, we created mutations in PDZ1 and PDZ2 to convert the PDZ12 tandem of

SAP97 from a class I to class II. We mutated histidine 256 and 351 (corresponding to PDZs 1 and 2, respectively) to valine (SAP97 H256V H351V) (Fig. 3.10A, B).

The SAP97 mutants were first examined biochemically by using a GST pull-down assay. The N-terminus (1-374 residues) of wild-type (SAP97 C1) and mutant (SAP97 C2, H256V H351V) SAP97 were fused to glutathione-S-transferase (GST). Recombinant proteins were purified from bacteria and incubated with extracts from HEK293 cells expressing Kv1.1 or Flag-β2AR. The purified class I GST-SAP97 (GST-SAP97 (1-374) C1), but not GST alone or class II GST-SAP97 (GST-SAP97 (1-374) C2), pulled down Kv1.1 and β2AR (Fig. 3.10C, D). These results suggested that the PDZ12 tandem of SAP97 directly interacted with Kv1.1 and β2AR, similar to a typical class I PDZ domain-ligand interaction.

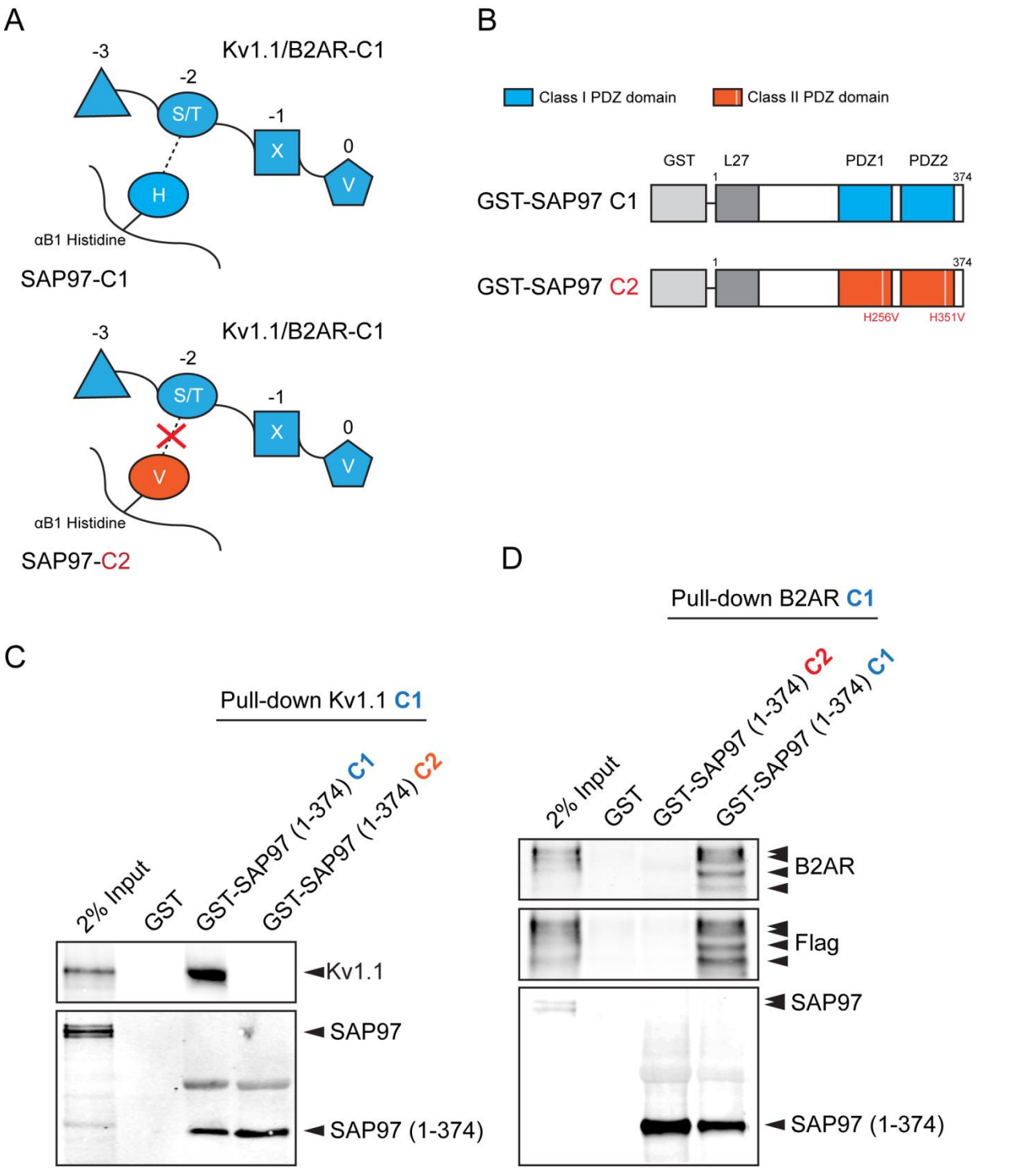


Figure 3.10 SAP97 interacts with Kv1.1 and β2AR through PDZ12 tandem.

(A) Representation of class I interaction between SAP97 and Kv1.1/β2AR. A histidine in PDZ1/2 domain is thought to bind threonine on the C-terminus PDZ binding motif of Kv1.1/ β2AR (upper panel). Mutations

at this position (PDZ1, H256V; PDZ2, H351V) could abolish this interaction (lower panel). **(B)** Schematic drawings of GST-tagged N-terminus (1-374 residues) of wild-type and mutant SAP97 were showing as GST-SAP97 (1-374) C1 and GST-SAP97 (1-374) C2, respectively. **(C, D)** GST pull-down experiments were performed with the indicated GST and GST-SAP97 fusion proteins, which were incubated with the lysates of HEK293 cells transfected with expression vectors for Kv1.1 or Flag-tagged β2AR (Flag-β2AR). Bound proteins were analyzed by SDS-PAGE, followed by western blotting with antibody against Kv1.1 (left panel), Flag and β2AR (right panel). GST-fusion proteins used are indicated by immunoblotting with antibody against the N-terminus of SAP97.

To determine more decisively whether Kv1.1 interact with the PDZ12 tandem of SAP97, we generated compensatory mutations in the PDZ domains of SAP97 and the C-terminus of Kv1.1. In addition to the constructs for truncated class I and class II SAP97 we used in the previous assay, we also generated constructs expressing GST-tagged full length SAP97 (Fig 3.11B). In the case of Kv1.1, we created point-mutations at the C-terminal class I PDZ binding motif to convert the PDZ/ligand interaction from a class I (TDV) to class II (FPV) (Fig 3.11A, B).

We applied these compensatory mutants to the design of a PDZ/ligand class switch model. Wild-type SAP97 PDZ12 tandem (referred to as SAP97 C1) harbors two class I PDZ domains characterized by the presence of a histidine residue at position 256 and 351. While introduction of a compensatory mutation in Kv1.1 (threonine 493 to Phenylalanine) could convert the class I PDZ domain binding motif into a class II PDZ domain binding motif and reconstitutes the Kv1.1-C2/SAP97-C2 interaction.

To test this model, we applied the mutants for GST-pull assay. Full length SAP97 (GST-SAP97 C1 (1-893), GST-SAP97 C2 (1-893)) and truncated SAP97 (GST-SAP97 C1 (1-374), GST-SAP97 C2 (1-374)) were fused with GST and purified from bacteria. GST fusion protein, or GST alone, immobilized on beads was incubated with extracts from HEK293 cells expressing either wild-type Kv1.1 or mutant Kv1.1 (T493F). We found that wild-type Kv1.1 C1 interact with both full length and truncated class I SAP97, but not with class II SAP97 (Fig. 3.11C). However, the point mutation in Kv1.1 C-terminus failed to disrupt the interaction with SAP97 C1 (either full length or truncated), or reconstitute interaction with SAP97 C2 (either full length or truncated) (data was not shown). Therefore, we created another point mutation at -1 position of Kv1.1 (T493F, D494P), referred to as Kv1.1 C2, to generate a class II PDZ binding motif, which has been shown in another PDZ/ligand class switch model to study the interaction between Stargazin and PSD-95 (Schnell et al., 2002) (Fig. 3.11A). We found that in contrast to Kv1.1 C1,

Kv1.1 C2 could only bind SAP97 with class II PDZ domains (Fig. 3.11C). These results confirmed the class I PDZ/ligand interaction between SAP97 PDZ12 tandem and Kv1.1, and suggested the compensatory mutants could reconstitute a class II PDZ/ligand interaction between SAP97 C2 and Kv1.1 C2.

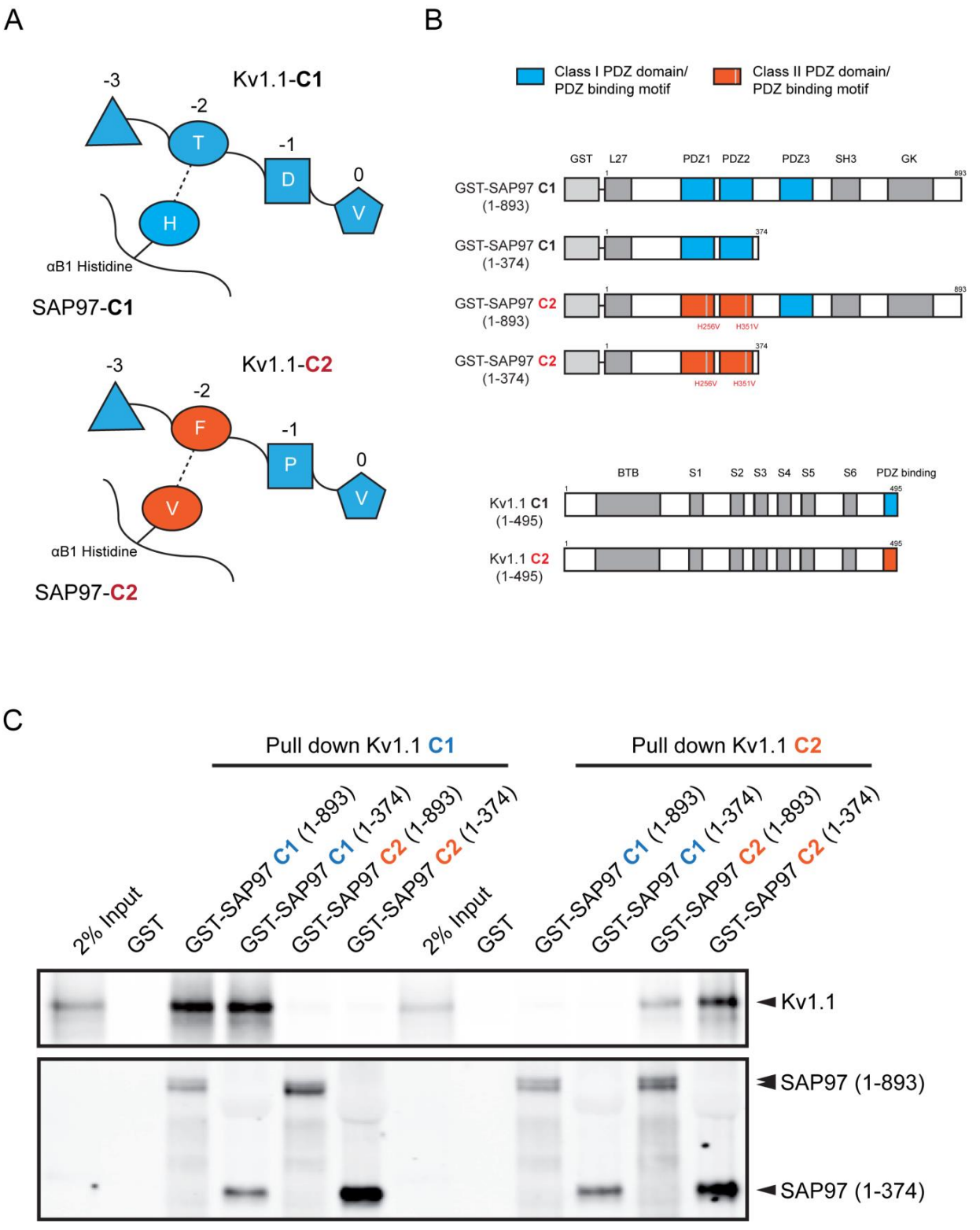

A
Kv1.1-C1
-3
-2
-1
0
T
D
V
H
αB1 Histidine
SAP97-C1
Kv1.1-C2
F
P
V
αB1 Histidine
SAP97-C2
B
Class I PDZ domain/ PDZ binding motif
Class II PDZ domain/ PDZ binding motif
GST
L27
PDZ1
PDZ2
PDZ3
SH3
GK
GST-SAP97 C1 (1-893)
GST-SAP97 C1 (1-374)
GST-SAP97 C2 (1-893)
GST-SAP97 C2 (1-374)
H256V
H351V
BTB
S1
S2
S3
S4
S5
S6
PDZ binding
Kv1.1 C1 (1-495)
Kv1.1 C2 (1-495)
C
Pull down Kv1.1 C1
Pull down Kv1.1 C2
2% Input
GST
GST-SAP97 C1 (1-893)
GST-SAP97 C1 (1-374)
GST-SAP97 C2 (1-893)
GST-SAP97 C2 (1-374)
Kv1.1
SAP97 (1-893)
SAP97 (1-374)

Figure 3.11 PDZ/ligand class switch experiment by using compensatory mutants showed reconstitution of Kv1.1/SAP97 interaction.

(A) Representation of a PDZ/ligand class switch model. Wild-type SAP97 PDZ12 tandem (referred to as SAP97 C1) has two class I PDZ domains with a histidine residue at position 256 and 351, respectively, which are required for the interaction with a threonine residue at position 493 (-2) of Kv1.1 class I PDZ domain binding motif (referred to as Kv1.1 C1). Mutations of the critical histidine residues to valine should convert SAP97 PDZ12 tandem from class I to class II (referred to as SAP97 C2). While introduction of a compensatory mutation in Kv1.1 (threonine 493 to Phenylalanine, referred to as Kv1.1 C2) should convert the class I PDZ domain binding motif into a class II PDZ domain binding motif and reconstitutes the Kv1.1-C2/SAP97-C2 interaction. **(B)** Schematic drawings of GST-tagged truncated class I and class II SAP97 (GST-SAP97 C1 (1-374), GST-SAP97 C2 (1-374)), GST-tagged full length class I and class II SAP97 (GST-SAP97 C1 (1-893), GST-SAP97 C2 (1-893)) and Kv1.1 with class I PDZ domain binding motif (Kv1.1 C1 (1-495)) or with class II PDZ domain binding motif (Kv1.1 C2 (1-495)). **(C)** Full length SAP97 (GST-SAP97 C1 (1-893), GST-SAP97 C2 (1-893)) and truncated SAP97 (GST-SAP97 C1 (1-374), GST-SAP97 C2 (1-374)) were fused with GST and purified from bacteria. GST fusion protein, or GST alone, immobilized on beads was incubated with extracts from HEK293 cells expressing either Kv1.1 C1 or Kv1.1 C2. Bound proteins were analyzed by SDS-PAGE, followed by western blotting with an antibody against Kv1.1. GST-fusion proteins used are indicated by immunoblotting with an antibody against the N-terminus of SAP97.

3.8 PDZ domains in SAP97 PDZ12 tandem shows different Kv1.1 binding affinity

It has been shown that single PDZ domain-mediated PDZ/ligand interaction is generally of modest specificity and affinity. As hundreds of PDZ domains and PDZ domain binding motifs exist in each mammalian genome, the promiscuous single PDZ/ligand interactions would not be compatible with the specific and fundamental roles of PDZ domain-containing proteins and their ligands in a diverse array of cellular processes. It has been implicated that the PDZ12 supramodule of DLG-MAGUKs provide the molecular basis for high affinity and specificity of PDZ/ligand binding, but the PDZ12 tandem would be biased towards a multimeric target with two identical PDZ binding motifs, such as K^+ channels or two NR2 subunits of NMDA receptors (Feng et al., 2009). Another study indicated that increasing the spacing between PDZ1 and

PDZ2 by lengthening the linker resulted in decreased binding affinity between PDZ12 tandem to K^+ channels and reduced capacity in the regulation of Kv1.4 clustering (Long et al., 2003).

To test whether a single PDZ domain in the PDZ12 tandem of SAP97 produces an impact on the affinity and specificity of Kv1.1/SAP97 interaction, we generated SAP97 expression constructs containing a single point-mutation either on PDZ1 (H256V) or PDZ2 (H351V), converting a single PDZ domain in the PDZ12 tandem from class I to class II (Fig. 3.12A). The mutant PDZ12 tandem were tagged with GST and applied for GST-pull down assay. I found that both of the single PDZ domain mutants showed reduced affinity to class I Kv1.1 compared to PDZ12 tandem with two class I PDZ domains, as well as to class II Kv1.1 compared to PDZ12 tandem with two class II PDZ domains (Fig 3.12B, C). To our surprise, the binding affinity of PDZ2 is higher than PDZ1, which may suggest functional differences in regulating Kv1.1 activity (Fig 3.12B, C).

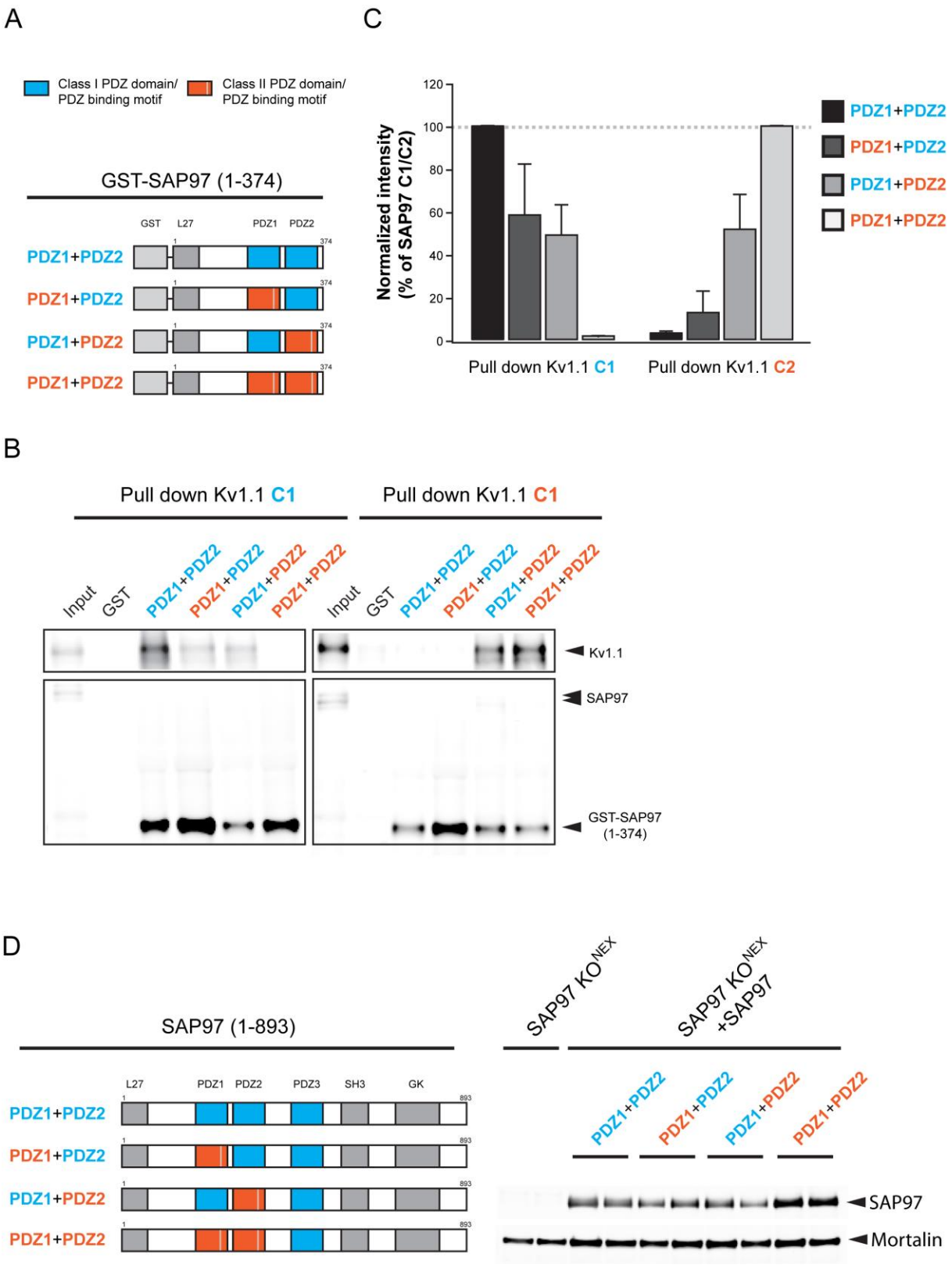

A
Class I PDZ domain/ PDZ binding motif
Class II PDZ domain/ PDZ binding motif
GST-SAP97 (1-374)
GST
L27
PDZ1
PDZ2
PDZ1+PDZ2
PDZ1+PDZ2
PDZ1+PDZ2
PDZ1+PDZ2
C
Normalized intensity (% of SAP97 C1/C2)
120
100
80
60
40
20
0
PDZ1+PDZ2
PDZ1+PDZ2
PDZ1+PDZ2
PDZ1+PDZ2
Pull down Kv1.1 C1
Pull down Kv1.1 C2
B
Pull down Kv1.1 C1
Pull down Kv1.1 C1
Input
GST
PDZ1+PDZ2
PDZ1+PDZ2
PDZ1+PDZ2
PDZ1+PDZ2
Input
GST
PDZ1+PDZ2
PDZ1+PDZ2
PDZ1+PDZ2
PDZ1+PDZ2
Kv1.1
SAP97
GST-SAP97 (1-374)
D
SAP97 (1-893)
L27
PDZ1
PDZ2
PDZ3
SH3
GK
PDZ1+PDZ2
PDZ1+PDZ2
PDZ1+PDZ2
PDZ1+PDZ2
SAP97 KO NEX
SAP97 KO NEX +SAP97
PDZ1+PDZ2
PDZ1+PDZ2
PDZ1+PDZ2
PDZ1+PDZ2
SAP97
Mortalin

Figure 3.12 PDZ domains in SAP97 PDZ12 tandem shows different Kv1.1 binding affinity.
(A) Schematic drawings of GST-tagged N-terminus (1-374 residues) of wild-type and mutant SAP97. Wild type PDZ domains (class I) were shown in Blue and PDZ domains with point mutations (class II) were shown in orange. **(B)** Wild-type and mutant truncated SAP97 were fused with GST and purified from bacteria. GST fusion protein, or GST alone, immobilized on beads was incubated with extracts from HEK293 cells expressing either Kv1.1 C1 or Kv1.1 C2. Bound proteins were analyzed by SDS-PAGE, followed by western blotting with antibody against Kv1.1. GST-fusion proteins used are indicated by immunoblotting with an antibody against the N-terminus of SAP97. **(C)** Quantification of the binding affinity of different SAP97 PDZ12 tandem mutants. The level of binding affinity to Kv1.1 C1 was normalized against the affinity level of wild-type SAP97 PDZ12 tandem. The level of binding affinity to Kv1.1 C2 was normalized against the affinity level of mutant SAP97 PDZ12 tandem containing two class II PDZ domains. **(D)** Schematic drawings of wild-type and mutant SAP97 containing one or two class II PDZ domains (left panel). Hippocampal neuronal cultures were prepared from P0 SAP97 KO^{NEX} mice, and letivirus-mediated overexpression of wild-type and mutant SAP97 were transduced on DIV4. Neurons were harvested on DIV17 and subjected to western blot with antibody against SAP97. Mortalin served as a loading control (right panel).

Then I tested the consequences of these mutations on protein function in hippocampal neurons. I generated constructs expressing full length wild-type or mutant SAP97 containing one or two point mutations (H256V and H351V) to convert one or two PDZ domains from class I to class II (Fig. 3.12D left panel). Lentivirus-mediated expression of SAP97 in SAP97 KO^{NEX} neurons could be detected by western blotting with an antibody against SAP97. The elevated expression level of SAP97 suggested the high efficiency of virus transduction and expression constructs delivery (Fig. 3.12 D right panel).

To characterize whether the reconstitution of Kv1.1/SAP97 interaction was associated with changes in regulation of NE-induced Kv1.1 internalization, compensatory mutants of Kv1.1 and SAP97 were introduced into SAP97 KO^{NEX} neurons. SAP97 constructs which was characterized in the previous experiment were delivered by lentivirus on DIV4. In the case of Kv1.1, bicistronic AAV vectors with shKv1.1, efficiently knocking down endogenous Kv1.1, and Kv1.1 cDNA, expressing class I or class II Kv1.1, were transduced on DIV 7. Co-expression of Kv1.1 C1 and SAP97 C2 resulted in an impairment of Kv1.1 internalization after NE treatment, which was identical to that obtained with Kv1.1 D4 (C-terminus deleted), suggesting that the mutation interfered with the function of Kv1.1/SAP97 complex (Kv1.1 C1+SAP97 (PDZ1_C1, PDZ2_C1) control, n=4: 100 ± 0.47% vs. Kv1.1 C1+SAP97 (PDZ1_C2, PDZ2_C2) NE, n=4: 111.13 ±

3.69%, p=0.054, .Fig. 3.13A upper panel). However, SAP97 containing a single class I PDZ domain, either PDZ1 or PDZ2, produced the effect of wild-type SAP97 with two PDZ II domains, in mediating NE-induced internalization of Kv1.1 C1 (Kv1.1 C1+SAP97 (PDZ1_C1, PDZ2_C1) control, n=4: 100 ± 0.47% vs. Kv1.1 C1+SAP97 (PDZ1_C2, PDZ2_C1) NE, n=3: 80.95 ± 0.057%, p<0.001; vs. Kv1.1 C1+SAP97 (PDZ1_C1, PDZ2_C2) NE, n=3: 67.89 ± 4.96%, p<0.05; vs. Kv1.1 C1+SAP97 (PDZ1_C1, PDZ2_C1) NE, n=6: 78.36 ± 10.21%, p<0.01. Fig. 3.13A upper panel). In striking contrast, co-expression of Kv1.1 C2 together with SAP97 bearing compensatory mutations enabled the surface removal of Kv1.1 C2. There was a reduction of surface Kv1.1 C2 after NE application. This occurred for SAP97 bearing a single compensatory mutation in PDZ1 or PDZ2, and for SAP97 with double compensatory mutation in both domain of PDZ12 tandem (Kv1.1 C2+SAP97 (PDZ1_C1, PDZ2_C1) control, n=6: 100 ± 4.01% vs. Kv1.1 C2+SAP97 (PDZ1_C1, PDZ2_C1) NE, n=6: 107.60 ± 8.13%, p=0.40; vs. Kv1.1 C2+SAP97 (PDZ1_C2, PDZ2_C1) NE, n=3: 85.78 ± 0.057%, p<0.05; vs. Kv1.1 C2+SAP97 (PDZ1_C1, PDZ2_C2) NE, n=3: 67.12 ± 3.12%, p<0.05; vs. Kv1.1 C2+SAP97 (PDZ1_C2, PDZ2_C2) NE, n=5: 67.31 ± 4.39%, p<0.001. Fig. 3.13A, lower panel).

These results indicated the crucial role of Kv1.1/SAP97 interaction in regulating NE-induced Kv1.1 internalization. Although the affinity was reduced with a single PDZ domain interaction, constructs with only PDZ1 or 2 being able to interact with Kv1.1 were sufficient to functionally rescue NE-induced Kv1.1 surface removal.

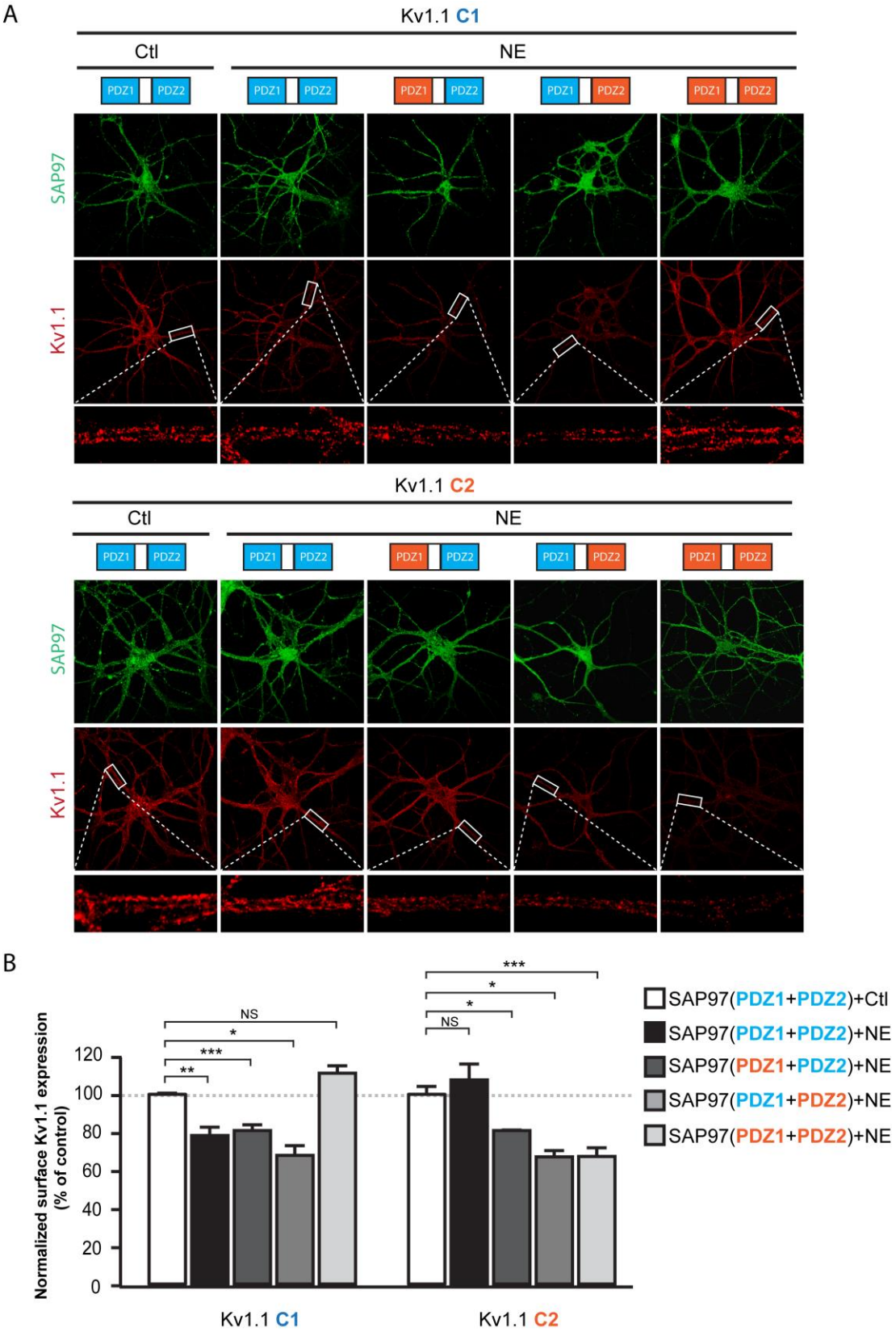

A
Kv1.1 C1
Ctl
NE
PDZ1
PDZ2
SAP97
Kv1.1
Kv1.1 C2
Ctl
NE
PDZ1
PDZ2
SAP97
Kv1.1
B
Normalized surface Kv1.1 expression (% of control)
120
100
80
60
40
20
0
NS
*
***
**
***
*
*
NS
Kv1.1 C1
Kv1.1 C2
SAP97(PDZ1+PDZ2)+Ctl
SAP97(PDZ1+PDZ2)+NE
SAP97(PDZ1+PDZ2)+NE
SAP97(PDZ1+PDZ2)+NE
SAP97(PDZ1+PDZ2)+NE

Figure 3.13 Reconstruction of Kv1. SAP97 interaction by compensatory mutations recues impairment of NE-induced Kv1.1 internalization in SAP97 KO^{NEX} neurons.

(A) Co-expression of Kv1.1 C1 (upper panel) or C2 (lower panel) together with various constructs of SAP97 bearing one or two point mutations (H256V or H351V) in SAP97 KO^{NEX} neurons. The point mutations should convert single or both PDZ domains of SAP97 PDZ12 tandem from class I to class II. Wild-type PDZ domains (class I) were shown in Blue and PDZ domains with point mutations (class II) were shown in orange. Expression of wild-type SAP97, or SAP97 bearing a compensatory mutation in either PDZ1 (H256V) or PDZ2 (H351V) rescued the impairment of NE-induced internalization of class I Kv1.1. In contrast, the internalization of class II Kv1.1 was only mediated by SAP97 containing class II PDZ domains. (B) Summary graph of surface Kv1.1 expression after NE treatment in SAP97 KO^{NEX} neurons co-expressed with compensatory mutants of Kv1.1 and SAP97 in panel A, normalized to untreated SAP97 KO^{NEX} neurons co-expressed with wild-type SAP97 together with class I/II Kv1.1.

3.9 PKA activity is required for NE-induced Kv1.1 internalization

The cAMP-dependent protein kinase (PKA) signaling transduction pathway has been suggested to play an important role in the modulation of the brain. It has been shown that the signaling scaffold A-kinase anchoring protein 5 (AKAP5) binds to the SH-GK domain of SAP97, the adenylyl cyclase 5/6 and its target PKA (Colledge et al., 2000). Activation of PKA results in phosphorylation of Kv1.1 at its C-terminal domain (S446), leading to its rapid translocation into the plasma membrane (Winklhofer et al., 2003). The complex scaffold by AKAP5 would ensure signaling specificity, such that the signaling transduction cascade from β2AR-activated Gαs to adenylate cyclase-dependent cAMP production, leading to PKA-mediated Kv1.1 phosphorylation, would be tethered together in one complex.

To test this hypothesis, I applied NE on hippocampal neurons pretreated with different kinase inhibitors, and then analyzed the internalization of Kv1.1. I found that the NE-triggered surface Kv1.1 reduction was prevented in the presence of the selective PKA inhibitor KT5720 (control vs. NE+KT5720, n=7; 109.96 ± 9.62%, p=0.42; Fig. 3.14A, B), but not by selective Mitogen-activated protein (MAP) kinase kinase (MKK) inhibitor U0126 (1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene) (control vs. NE+U0126, n=12; 80.65 ± 4.96%, p<0.05; Fig. 3.14A, B). These results suggested that the effect of NE in regulating Kv1.1 internalization needs the activity of PKA and not as previously shown in HEK293 cells, the surface delivery.

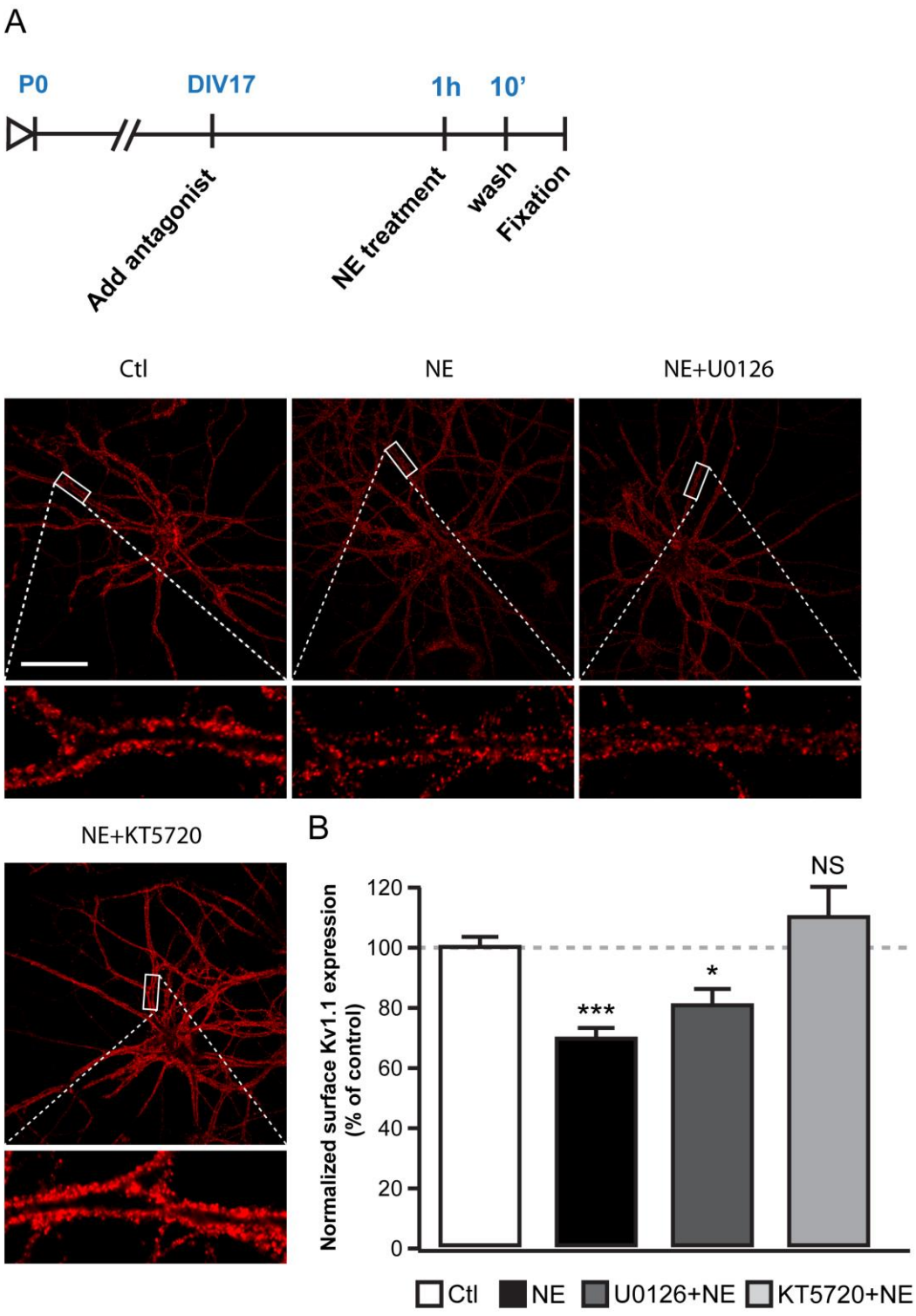


Figure 3.14 NE-mediated Kv1.1 internalization requires PKA activity.

(A) Hippocampal neurons were pretreated with the selective PKA inhibitor KT5720 and the selective MAP kinase kinase inhibitor U0126, followed by NE treatment (20 µM, 10 min) and cell surface labeling with Kv1.1 extracellular antibody. The lower panels present a magnified view of a dendritic stretch, which was

analyzed. **(B)** Summary graph of surface Kv1.1 expression in neurons treated with different kinase inhibitors in panel A, normalized to untreated control neurons.

3.10 Kv1.1-Ser 446 is necessary for NE-induced Kv1.1 internalization

It is well known that activation of β adrenergic receptors by NE binding causes a rise of the intracellular concentration of the second messenger cAMP. Downstream effectors of cAMP include PKA and EPAC (Wang et al., 2004).

To verify this effect in the hippocampus, we incubated hippocampal slices in NE-containing medium (20µM, 10 min) and assayed for the change in PKA activity. It has been shown that NE-mediated β-AR activation triggers the phosphorylation of Ser-845 of AMPA receptor subunit GluA1, which is required for one form of NE-facilitated LTP (Hu et al., 2007). I also found that NE treatment caused an increase in the phosphorylation of GluA1 at Ser845 in hippocampal slices (Fig. 3.14A). In addition, elevated levels of PKA substrate phosphorylation, detected by a phospho-S/T antibody, suggested an enhancement of PKA activity in NE-treated hippocampal slices (Fig. 3.14A).

To determine whether Kv1.1 is the target of PKA phosphorylation and whether the phosphorylation is required for Kv1.1 desensitization, we replaced endogenous Kv1.1 with a mutant, changing Ser-446 to an alanine residue (Kv1.1 S446A). It has been reported that the phosphorylating of this site brought a specific band shift of Kv1.1 in western blots, while mutating of this site to alanine residue abolished the forskolin-induced band shift, suggesting the potential PKA-phosphorylation of Kv1.1 Ser-446 (Winklhofer et al., 2003). Using bicistronic lentivirus-mediated Kv1.1 replacement strategy, endogenous Kv1.1 was efficiently knocked-down by shKv1.1 and substituted with either wild-type Kv1.1 or Kv1.1 S446A. First, I found that the phosphorylation-deficient Kv1.1 (Kv1.1 S446A) replacement did not produce any effect on the surface expression. Second, NE treatment induced the internalization of wild-type Kv1.1 (Kv1.1 WT replacement control, n=7: 100 ± 3.50% vs. Kv1.1 wild-type replacement NE, n=7: 76.46 ± 2.94%, $p<0.0001$, Fig. 3.15B, C), but exhibited no effect on hippocampal neurons with Kv1.1 S446A (Kv1.1 S446A replacement control, n=3: 100 ± 7.03% vs. Kv1.1 S446A replacement NE, n=3: 113.87 ± 14.77%, $p=0.46$, Fig. 3.15B, C). These results suggested that the phosphorylation of Kv1.1-Ser 446 was required for NE-induced Kv1.1 internalization.

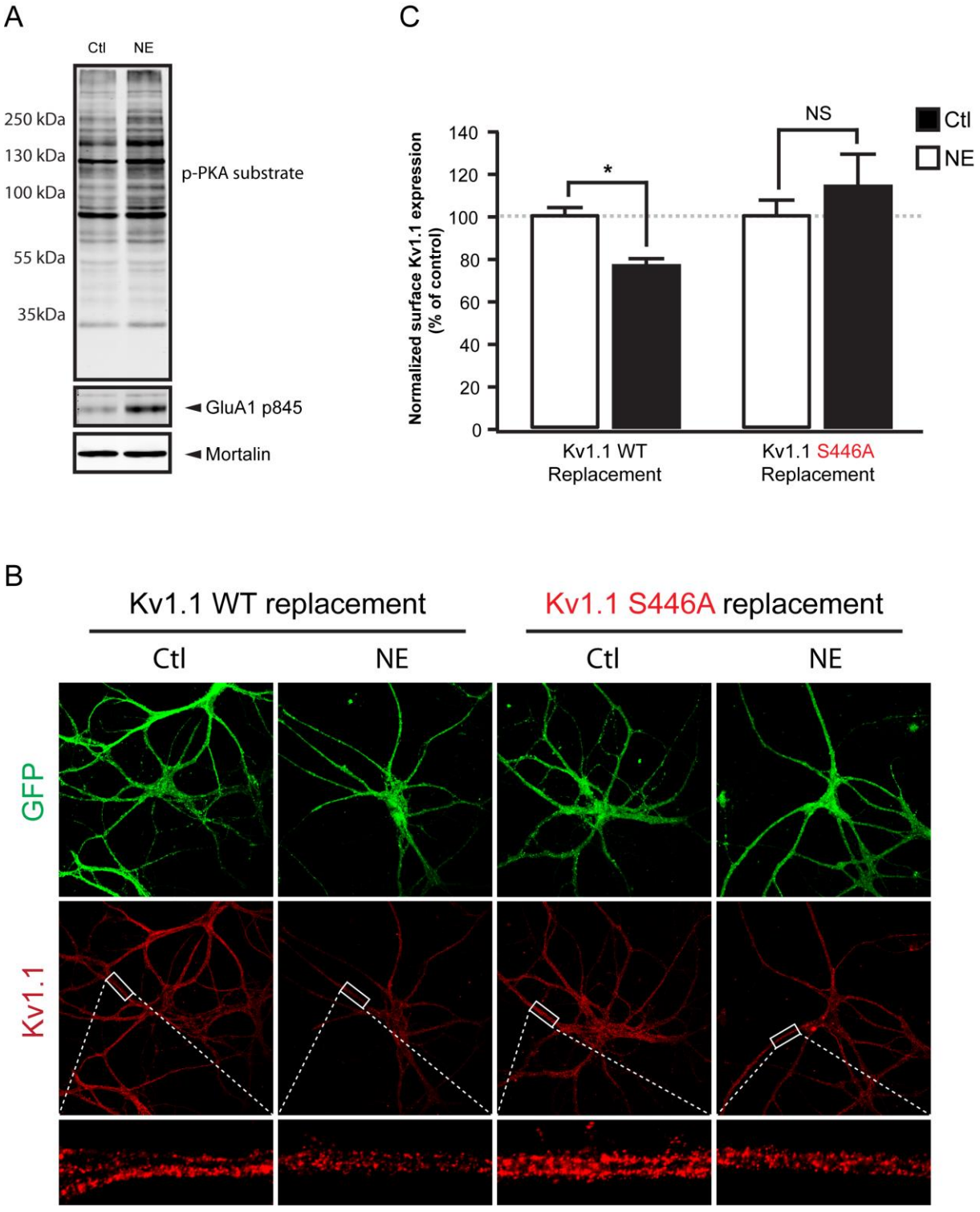

A
Ctl
NE
250 kDa
130 kDa
100 kDa
55 kDa
35kDa
p-PKA substrate
GluA1 p845
Mortalin
C
Normalized surface Kv1.1 expression (% of control)
140
120
100
80
60
40
20
0
*
NS
Ctl
NE
Kv1.1 WT Replacement
Kv1.1 S446A Replacement
B
Kv1.1 WT replacement
Kv1.1 S446A replacement
Ctl
NE
Ctl
NE
GFP
Kv1.1

Figure 3.15 Kv1.1-Ser 446 is required for NE-induced Kv1.1 internalization

(A) NE treatment elevated PKA-activity in hippocampus. Hippocampal slices were incubated in NE-containing medium (20µM, 10 min) and assayed for the change in the phosphorylation of GluR1-Ser845 and PKA substrate. Mortalin served as a loading control. **(B)** Molecular replacement assay of Kv1.1 S446A. Wild-type Kv1.1 or Kv1.1 S446A was cloned into bicistronic AAV vector together with highly efficient shRNA to knock-down endogenous Kv1.1. We applied the molecular replacement strategy with these constructs on hippocampal neurons and tested NE-induced Kv1.1 internalization of Kv1.1. **(C)** Summary graph of NE-induced surface Kv1.1 expression reduction in neurons infected with different AAV-mediated Kv1.1 replacement in panel B, normalized to untreated neurons.

4 Discussion

4.1 Kv1.1 is targeting to both dendrites and axons in hippocampal neurons

Early studies have demonstrated that Kv1 channels were primarily targeted to axons (Bekkers and Delaney, 2001; Gu et al., 2003), and played a modulatory role of action potential generation (Lai and Jan, 2006). Recent study showed that the surface expression of Kv1.1 could be detected in dendrites in punctate structure and elevated by the mTOR inhibitor, rapamcin (Raab-Graham et al., 2006). Consisting with the previous results, we found Kv1.1 was prominently expressed in hippocampal neuron al dendrites (Fig 3.2A; Fig. 4.1A).

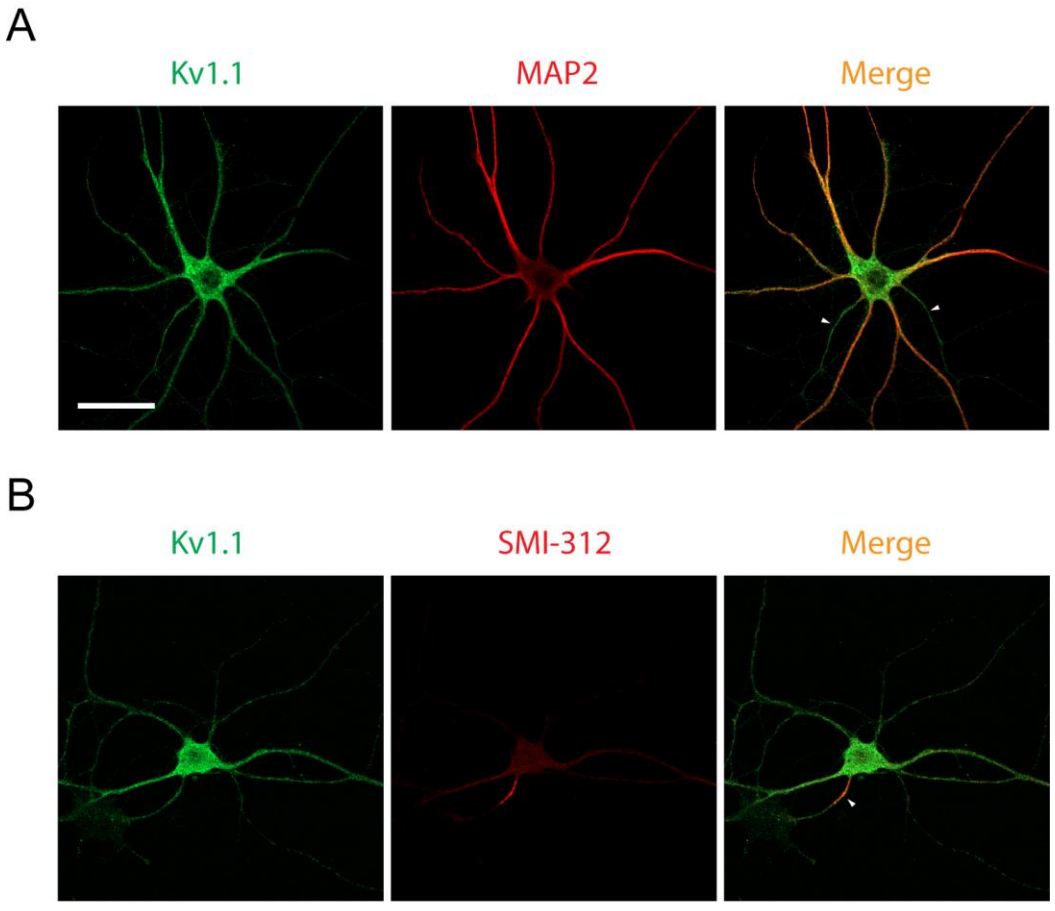


Figure 4.1 Kv1.1 is prominently expressed in dendrites of hippocampal neurons.
(A) Double immunostaining for Kv1.1 and MAP2 (dendritic marker). DIV17 primary hippocampal cultures were incubated with antibodies against Kv1.1 (green) and dendritic marker MAP2 (red). **(B)** Double immunostaining for Kv1.1 and SMI-312 (axonal marker). DIV17 primary hippocampal cultures were incubated with antibodies against Kv1.1 (green) and axonal marker SMI-312 (red). Colocalization can be seen as double-labeled regions in yellow in the merged images. Axons were indicated by arrowheads.

To characterize the subcellular localization of Kv1.1 in hippocampal neurons, we performed immunochemistry and colabeled Kv1.1 together with dendritic marker MAP2 (Fig. 4.1A) or axonal maker (Fig. 4.1B). We found the Kv1.1 immunereactivity was present on both MAP2-positive neuritis and SMI-312 neuritis, suggesting the dendritic and axonal localization of Kv1.1 in hippocampal neurons. We also found Kv1.1 could interact with a signaling scaffold protein SAP97 (Fig. 3.5A, B), which was preferentially expressed in neuronal dendrites (Waites et al., 2009), indicating the modulatory role of SAP97 in dendritic excitability.

4.2 NE/β2-AR signaling modulates neuronal excitability through Kv1.1

It has been reported that the activation of β-adrenergic receptor could enhance neuronal excitability through potassium channel inactivation. Electrophysiology study showed that activation of β-adrenergic receptor by increase intracellular Protein Kinase A (PKA) or Protein Kinase C (PKC) could lead to a subsequent depolarizing shift in activation curve for dendritic K^+ channels, and therefore enhance the amplitude of back-propagating dendritic action potentials (Hoffman and Johnston, 1999). Further study characterized an A-type potassium channel, Kv4.2, which is dully regulated by PKA and PKC through a common downstream pathway involving MAPK and responsible for the β-adrenergic receptor-activated increase in the amplitude of back-propagating action potentials in distal dendrites (Yuan et al., 2002). However, it has remained elusive how β-adrenergic receptor activation regulating potassium channels and modulate neuronal excitability. By using “molecular replacement” and “compensatory mutation” approaches, we introduced Kv1.1 and SAP97 with point-mutation into hippocampal neurons, which could interrupt or reconstitute Kv1.1/SAP97/β2AR interaction. We found both Kv1.1 and β2AR could interact with SAP97 PDZ12 tandem with their C-terminus PDZ domain-binding motif

(Fig. 3.10C, D), and these interactions are required for the β2-AR activation mediated Kv1.1 internalization. These results suggested the activation of β2-AR by norepinephrine could induce the desensitization of potassium channel and modulate neuronal excitability by triggering the internalization of Kv1.1.

4.3 SAP97 serves as a signaling scaffolding linking β2AR to Kv1.1 internalization

The interactions between DLG-MAKGUKs and Kv1 channels have long been characterized. Structural and mutational analyses revealed SAP97 and other MAGUKs bind to a consensus PDZ-binding sequence (xS/TxV) at the COOH terminus of Kv1 α subunits via either of the first two of the three PDZ domains located near the NH2 terminus (Kim and Sheng, 1996; Tiffany et al., 2000). In heterozygous system, PSD-95 and SAP97 has been shown to regulate the surface expression of Kv1 channels differently (Tiffany et al., 2000). PSD-95 could interact with Kv1 channel α subunits and facilitate the surface expression of several Kv1 channels, while SAP97 could cluster Kv1 channels and prevent its surface expression (Tiffany et al., 2000).

Our data obtained in hippocampal neurons revealed a different role of PDZ domain-PDZ binding motif interaction in regulating Kv1 channel surface trafficking. In primary hippocampal neuronal cultures, the surface expression of Kv1.1 was not altered by deleting the C-terminus PDZ domain binding motif (Kv1.1 D4 (Fig. 3.6A, right panel), or conditional knocking-out SAP97 (Fig 3.7 A). In contrast, Kv1.1 C-terminus PDZ binding motif/SAP97 PDZ domain interaction was required for the NE-induced Kv1.1 internalization. The NE-induced β2-AR-dependent Kv1.1 internalization was impaired in the Kv1.1-D4 replacement and SAP97 conditional knockout neurons (Fig. 3.6; Fig. 3.7). These results suggested that in hippocampal neurons, not the surface expression of Kv1.1 required C-terminus PDZ binding motif/SAP97 PDZ domain interaction, but rather the modulation.

It has been reported that PSD-95 could regulate the signaling complex of β2-AR/GluR1/PKA, promote phosphorylation and surface expression of GluR1 and therefore modulate AMPA receptor function (Joiner et al., 2010). Besides β2-AR, the interaction between PSD-95 and β1-AR has also been well clarified. The interaction between β1-AR and PSD-95 has long been identified in yeast two-hybrid system and HEK-293 cells (Hu et al., 2000). PSD-95 can inhibit the agonist-induced β1-AR endocytosis and stabilize the receptor at the surface, but have no

effect on receptor desensitization or receptor-induced cAMP accumulation (Hu et al., 2000). Further evidences demonstrated that β1-AR association with PSD-95 is regulated by G protein-coupled receptor kinase 5 (GRK5), and the phosphorylation of β1-AR by GRK5 decreases its interaction with PSD-95 (Hu et al., 2002). However, we found the impairment of NE-induced internalization of Kv1.1in SAP97 knockout neurons could only be rescued by overexpression of PSD-93, but not by PSD-95 or SAP102 (Fig. 3.8). These results indicated a different role of SAP97, than PSD-95, in orchestrating G-protein coupled receptor signaling.

4.4 PDZ12 tandem is required for SAP97-coordinated signaling complex of β2-AR/SAP97/Kv1.1

To determine whether a direct interaction between SAP97 and Kv1.1 could orchestrate NE/β2AR signaling and regulate Kv1.1 surface trafficking, we mutated both SAP97 and Kv1.1 in a complementary manner, allowing them to reconstitute the specific interaction and bind uniquely with each other (Fig. 3.10A, B). A point mutation in the C-terminus PDZ domain binding motif of Kv1.1 could not bind to class I PDZ domain-containing proteins (Fig 3.10C), and therefore not be internalized after NE treatment normally observed with wild-type SAP97 (Fig. 3.13). However, when this mutant was expressed with SAP97 constructs bearing compensatory point mutations, which could reconstitute Kv1.1 binding, the internalization was fully rescued. This data identified the interaction between SAP97 (or other DLG-MAGUKs) and Kv1.1 as an important aspect in the function of both proteins.

It has been suggested that single PDZ domain-mediated interaction is of modest specificity and affinity (Feng et al., 2009). In this case, the promiscuous single PDZ/ligand interactions would not be compatible with the specific and fundamental roles of PDZ domain-containing proteins and their ligands in multiple functions. So the PDZ12 tandem would be biased towards a multimeric target with two identical PDZ binding motifs to increase the binding specificity and affinity. Our data suggested that SAP97 PDZ1 and PDZ2 domains are both involved in SAP97/Kv1.1 interaction, but binding affinity of PDZ2 is higher than PDZ1 (Fig. 3.12B, C). However, the higher Kv1.1 binding affinities of PDZ2 was not associated to different properties in regulating NE-induced Kv1.1 internalization when co-expressed with SAP97 constructs bearing compensatory point mutations (Fig. 3.13). These results are consistent with previous studies (Feng et al., 2009), suggesting the PDZ domains of SAP97 PDZ12 tandem have

identical affinity and specificity in orchestrate NE/β2AR signaling and regulate Kv1.1 surface trafficking.

4.5 PKA phosphorylation of Kv1.1-Ser 446 is required for NE-induced Kv1.1 internalization

An AKAP5-coordinated signaling scaffolding complex containing SAP97, the adenylyl cyclase 5/6 and its target PKA has been characterized (Colledge et al., 2000). Phosphorylation of Kv1.1 by PKA at its C-terminal domain (S446) could cause the rapid translocation of Kv1.1 into plasma membrane in HEK293 cells (Winklhofer et al., 2003). Our results showed that NE-induced reduction in Kv1.1 surface expression is blocked by KT-5720, a selective PKA blocker (Fig. 3.14), and a phosphorylation-deficient mutant of Kv1.1 (Kv1.1 S446A) was not internalized after NE-treatment (Fig. 3.15B, C). However, in HEK293 cells, phosphorylation of this site was shown to increase surface expression (Winklhofer et al., 2003), suggesting the opposite function of Kv1.1-Ser 446 phosphorylation in neurons and heterologous expression system. Since β2-AR are only known to act through Gαs and adenylate cyclase causing PKA activation, we proposed that the complex scaffold by AKAP5 would ensure signaling specificity, such that the signaling transduction cascade from β2AR-activated Gαs to adenylate cyclase-dependent cAMP production, leading to PKA-mediated Kv1.1 phosphorylation, would be tethered together in one complex.

We postulated the following model (Fig. 4.2): SAP97 links β2-adrenergic receptor with Kv1.1 and signal transduction pathway, which leads to the suppression of Kv1.1 by internalization in hippocampal neurons. Activation of β2-adrenergic receptor by norepinephrine stimulation triggers the dissociation of Gαs, which activates adenylate cyclase and leads to an increase of cAMP synthesis, followed by an enhancement of PKA activity. Phosphorylation of Kv1.1 at its C-terminus (Ser 446) by PKA triggers its internalization. Such a model relies heavily on the central role of SAP97 mediated interaction with β2AR and Kv1.1 by its PDZ12 tandem.

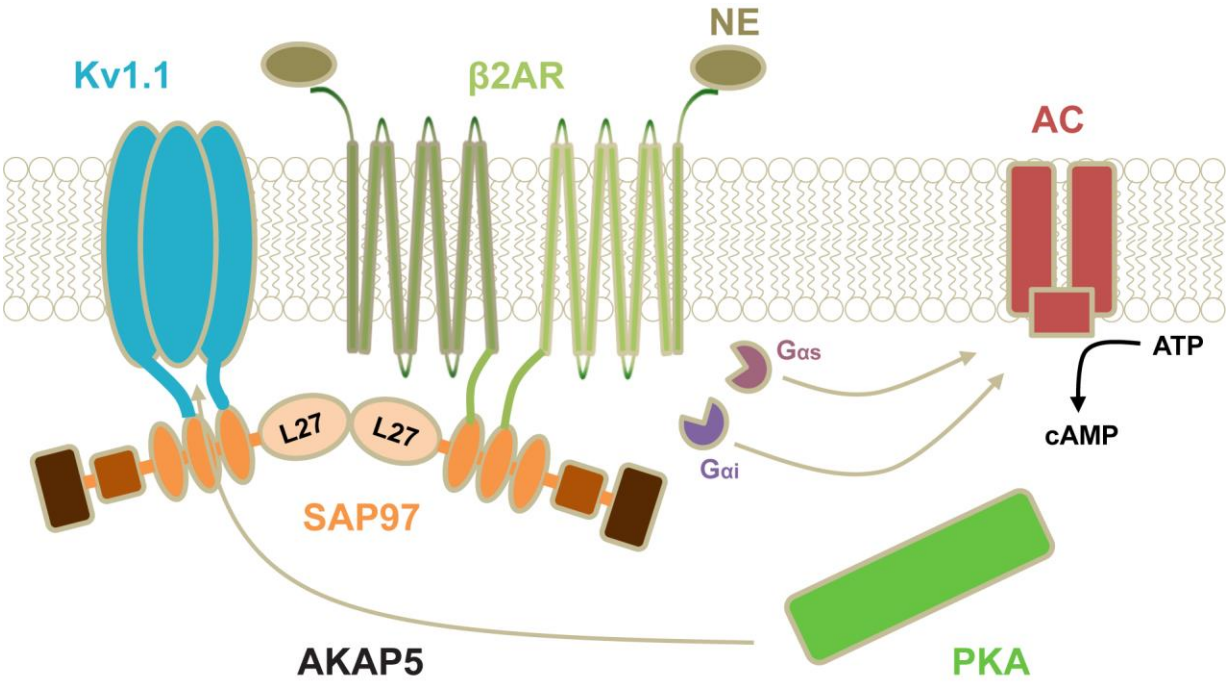


Figure 4.2 Schematic presentation of postulated b2AR signaling complex and Kv1.1 internalization.

Our data indicated a function of SAP97 to orchestrate NE/β2-AR signaling and regulate Kv1.1 surface trafficking. Activation of β2-adrenergic receptor by norepinephrine stimulation triggers the dissociation of Gαs, which activates adenylate cyclase and leads to an increase of cAMP synthesis, followed by an enhancement of PKA activity. PKA phosphorylates the C-terminus of Kv1.1 (Ser 446) and triggers its internalization. SAP97 PDZ12 tandem-mediated interaction with β2AR and Kv1.1 plays a key role in this model.

5 Reference

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