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The Role of SAP97 Isoforms in Regulating Synaptic Function in Hippocampal Neurons

Dong Li

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy. Department of Physiology. The University of Auckland, 2011
Abstract

SAP97 is a member of the discs-large family of membrane-associated guanylate kinases (MAGUK), and is a multidomain scaffold protein implicated in the forward trafficking and synaptic localization of NMDA- and AMPA-type glutamate receptors. Specifically, SAP97 directly binds the GluR1 subunit of AMPA receptors and NR2 subunits of NMDA receptors and participates in their forward trafficking from the Golgi network to the plasma membrane. Alternative splicing of SAP97 in the N-terminus gives rise to palmitoylated αSAP97 and L27-domain containing βSAP97 isoforms, while insertions between the SH3 and GUK domains results in further isoforms containing the I3, I2, I4 and/or I5 inserts. This study aimed to investigate the effects of SAP97 isoforms on synaptic transmission and plasticity in hippocampal neurons. It is known that the synaptic insertion of GluR1-containing AMPA receptors is critical for synaptic plasticity, under the control of NMDA receptors. However, the mechanisms responsible for GluR1 insertion and retention at the synapse are unclear. Whether SAP97 plays a role in scaffolding GluR1 at the postsynaptic membrane is controversial, attributable to its expression as a collection of alternatively spliced isoforms with ill-defined spatial and temporal distributions. In the present study, we have used electrophysiology and imaging to demonstrate that postsynaptic, N-terminal splice isoforms of SAP97 directly modulate the levels and function of synaptic AMPARs and NMDARs. Overall, αSAP97-I3 and βSAP97-I3/I2 differentially influence the subsynaptic localization and dynamics of AMPARs by creating binding sites for GluR1-containing receptors within synaptic and extrasynaptic subdomains. With regards to synaptic plasticity, i.e. long-term potentiation (LTP) and depression (LTD), we found that both α- and β-isoforms of SAP97-I3 impair LTP but enhance LTD via independent isoform-specific mechanisms. By recording from pairs of synaptically coupled hippocampal neurons, we show that αSAP97-I3 occludes LTP by enhancing the levels of postsynaptic AMPA receptors, while βSAP97 blocks LTP by reducing the synaptic localization of NMDA receptors. Examination of the surface pools of AMPA and NMDA receptors indicates that αSAP97-I3 selectively regulates the synaptic pool of AMPA receptors, whereas βSAP97-I3 and βSAP97-I2 regulate the extrasynaptic pools of both AMPA and NMDA receptors. Knockdown of βSAP97-I3 increases the synaptic localization of both AMPA and NMDA receptors, showing that endogenous βSAP97-I3 restricts glutamate receptor expression at excitatory synapses. This isoform-dependent differential regulation of synaptic versus extrasynaptic pools of glutamate receptors will determine how many receptors are available for the induction and the expression of synaptic plasticity. These results indicate that N-terminal splicing of SAP97 can regulate the ability of synapses to undergo plasticity by controlling the surface distribution of AMPA and NMDA receptors.
Acknowledgements

First and foremost I would like to thank my supervisor, Dr. Johanna Montgomery. Thank you for your tireless enthusiasm to science. It has been an exciting and challenging nearly five years in your lab and I am grateful for all that you have done for laying the foundation for my career.

Special thanks must be made to my parents for always encouraging and supporting me throughout life, so that I can reach my full potential.

Thank you also to my lab colleagues past and present; Juliette, Lin-Chien, Louise, Janie, Chantelle, Charlotte, Meagan, Kevin, David, Paula, Manja, Emma and Lucy. Thank you for your support and friendship.

Thank you to my previous colleagues Prof. Janusz Lipski, Michael, Kenny, Peter and Ji-Zhong. Thank you for your support and friendship.

Thank you to Prof. Alistair Gunn, for all of your help and support.

Finally, a special thank you to my wife Caiping and sons, Bill & Steven. Thank you for your strong support and enduring my PhD with me.
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<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>AKAP</td>
<td>A-kinase anchoring proteins</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-4-propionic acid</td>
</tr>
<tr>
<td>AMPAR</td>
<td>α-amino-3-hydroxy-4-propionic acid receptor</td>
</tr>
<tr>
<td>AP5</td>
<td>DL-2-amino-5-phosphonopentanoic acid</td>
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<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca²⁺/calmodulin dependent protein kinase II</td>
</tr>
<tr>
<td>CASK</td>
<td>calmodulin-associated serine kinase</td>
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<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione disodium salt hydrate</td>
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<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>C-terminus</td>
<td>carboxy-terminus</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DIV</td>
<td>days <em>in vitro</em></td>
</tr>
<tr>
<td>Dlg</td>
<td>Drosophila tumor suppressor discs large</td>
</tr>
<tr>
<td>EPSC</td>
<td>excitatory postsynaptic current</td>
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<td>excitatory postsynaptic potential</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<td>green fluorescent protein</td>
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<td>GK</td>
<td>guanylate kinase-like domain</td>
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<td>--------------</td>
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<tr>
<td>GluR</td>
<td>glutamate receptor</td>
</tr>
<tr>
<td>H</td>
<td>Hanks' Balanced Salt Solution</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks' Balanced Salt Solution</td>
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<tr>
<td>HEK293</td>
<td>Human Embryonic Kidney 293</td>
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<tr>
<td>HFS</td>
<td>high-frequency stimulation</td>
</tr>
<tr>
<td>I</td>
<td>ionotropic glutamate receptor</td>
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<td>iGluR</td>
<td>ionotropic glutamate receptor</td>
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<tr>
<td>K</td>
<td>potassium</td>
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<tr>
<td>K+</td>
<td>potassium</td>
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<td>L</td>
<td>low frequency stimulation</td>
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<tr>
<td>LFS</td>
<td>low frequency stimulation</td>
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<tr>
<td>LTD</td>
<td>long term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long term potentiation</td>
</tr>
<tr>
<td>M</td>
<td>membrane-associated guanylate kinase</td>
</tr>
<tr>
<td>MAGUK</td>
<td>membrane-associated guanylate kinase</td>
</tr>
<tr>
<td>MAP</td>
<td>microtubule associated protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>Mg2+</td>
<td>magnesium</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>N</td>
<td>sodium</td>
</tr>
<tr>
<td>Na+</td>
<td>sodium</td>
</tr>
<tr>
<td>NBM</td>
<td>neurobasal media</td>
</tr>
<tr>
<td>NBQX</td>
<td>FG 9202 disodium salt hydrate, 1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-bulfonamide disodium salt hydrate</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>N-terminus</td>
<td>amino-terminus</td>
</tr>
<tr>
<td>P</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PBST</td>
<td>0.25 % Triton in 1x PBS</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>PDZ</td>
<td>PSD95, Dlg and Zo-1</td>
</tr>
<tr>
<td>PKA</td>
<td>cyclic AMP-dependent protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>PP2B</td>
<td>protein phosphatise 2B</td>
</tr>
<tr>
<td>PPD</td>
<td>paired pulse depression</td>
</tr>
<tr>
<td>PPF</td>
<td>paired pulse facilitation</td>
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<tr>
<td>PPR</td>
<td>paired pulse ratio</td>
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<tr>
<td>PSD</td>
<td>postsynaptic density</td>
</tr>
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<td>PSD-93</td>
<td>postsynaptic density protein 93 kDa</td>
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<td>PSD-95</td>
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<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
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<tr>
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<td>ribonucleic acid</td>
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<td>ribonucleic acid</td>
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<tr>
<td>Src</td>
<td>sarcoma</td>
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<tr>
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<td>transmembrane AMPAR regulatory protein</td>
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<td>TARP</td>
<td>transmembrane AMPAR regulatory protein</td>
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<td>U</td>
<td>ultraviolet</td>
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Chapter 1. General Introduction

1.1. Overview

Synapses are cell-cell contact sites responsible for communication between the 100 billion neurons in the brain. Glutamatergic synapses within the hippocampus, the part of the brain very important in learning & memory, undergo synaptic plasticity, the cellular basis underlying learning and memory. Long term potentiation (LTP) and Long term depression (LTD) are two major forms of synaptic plasticity, causing synaptic strength to increase (LTP) or decrease (LTD). At glutamatergic synapses, N-methyl D-aspartate (NMDA) receptors act as ‘coincidence detectors’ to determine the direction of synaptic plasticity, i.e. LTP and LTD. The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are critical for LTP and LTD expression. Bound to AMPA and NMDA receptors are a family of membrane-associated guanylate kinase homologs (MAGUKs). These proteins are synaptic scaffolding proteins that act as central organisers of the postsynaptic density (PSD). MAGUKs are responsible for clustering many important molecules and channels including NMDA receptors and AMPA receptors in the PSD. Synapse-associated protein 97 (SAP97) is a primary member of MAGUK family, directly interacting with both AMPARs and NMDARs and regulating their function and trafficking to the synapse. Thus, SAP97 is thought to be a critical PSD-scaffolding protein involving in synaptic plasticity including LTP and LTD. SAP97 is alternatively spliced, producing multiple isoforms of underlying function. However, there are many neuronal functions of SAP97 that remain to be solved. Multi-discipline approaches including electrophysiology, imaging and molecular biology are necessary to reveal how SAP97 and its isoforms regulate synaptic function. The overall aim of this research is to understand how SAP97 contributes to physiological and possibly pathological function of synapses in the hippocampus.

1.2. The Hippocampus

A PubMed search of the word “hippocampus” yields over 100,000 publications, revealing the enormous amount of research performed in this part of the brain. The hippocampus is located inside the medial temporal lobe. The major components of hippocampus are areas CA1, CA3 and the dentate gyrus. Pyramidal neurons and granule cells are primary cells in areas CA1, CA3 and dentate gyrus respectively (Fig.1.1) (Guilherme et al., 2008). The entorhinal cortex is the major input and output of the
In processing information for explicit memory storage, the entorhinal cortex (EC) is a bidirectional bridge and has dual functions (Kandel et al., 2000). First, it is the main input to the hippocampus. The entorhinal cortex projects to the dentate gyrus via the perforant pathway and by this means provides the critical input pathway through which the information from the association cortices reaches the hippocampus (Kandel et al., 2000; Guilherme et al., 2008).

Figure 1-1 Anatomy of the hippocampus.

The wiring diagram of the hippocampus is traditionally presented as a trisynaptic loop. The major input is carried by axons of the perforant path, which convey polymodal sensory information from neurons in layer II of the entorhinal cortex to the dentate gyrus. Perforant path axons make excitatory synaptic contact with the dendrites of granule cells: axons from the lateral and medial entorhinal cortices innervate the outer and middle third of the dendritic tree, respectively. Granule cells project, through their axons (the mossy fibres), to the proximal apical dendrites of CA3 pyramidal cells which, in turn, project to ipsilateral CA1 pyramidal cells through Schaffer collaterals and to contralateral CA3 and CA1 pyramidal cells through commissural connections. The distal apical dendrites of CA1 pyramidal neurons receive a direct input from layer III cells of the entorhinal cortex (Guilherme et al., 2008).

Second, the entorhinal cortex is also the major output of the hippocampus. The information coming to the hippocampus from the polymodal association cortices and that coming from the hippocampus to association cortices converge in the entorhinal cortex.
3

(Kandel et al., 2000). Dentate gyrus granule cells receive input from the entorhinal cortex via the perforant pathway and project via the mossy fiber pathway onto CA3 pyramidal neurons (Guilherme et al., 2008). CA3 pyramidal cells then project via the Schaffer collateral pathway onto CA1 pyramidal neurons which are the primary output cells of the hippocampus (Fig.1.1). The hippocampus is one of the most important components for learning and memory in brain. In humans, the memory deficits might be closely related to the changes of synapses in the hippocampus. During normal aging, the numbers of primary hippocampal glutamatergic neurons counted stereologically in humans, monkeys or rats do not decline (including dentate gyrus, CA3 and CA1 principal cells). If primary hippocampal glutamatergic neuron numbers are not affected, why do old adults have memory deficits? One probable answer to this question is the changes at the synapse that result in altered mechanisms of plasticity (Burke & Barnes, 2010).

1.3. Glutamatergic Synapses

Glutamatergic synapses are excitatory synapses that utilise glutamate as their transmitter. Glutamate (Glu) is the principal excitatory neurotransmitter in the brain, and its interactions with specific membrane receptors are responsible for many neurologic functions, including cognition, memory, movement, and sensation (Lipton & Rosenberg, 1994). In the cerebral cortex and hippocampus, a great majority (80–90%) of the neurons use glutamate as a neurotransmitter and hence form excitatory synapses (Shinohara & Hirase, 2009). Neurons in the central nervous system receive excitatory synaptic input from glutamatergic neurons and inhibitory input from GABA releasing (GABAergic) interneurons. The balances between the glutamatergic synapses and inhibitory synapses are important to maintain synaptic functions. The proper balance between excitatory and inhibitory synapses is crucial for information processing and higher brain functions. Neurological disorders such as autism, schizophrenia and epilepsy are associated with an imbalance between excitatory and inhibitory synapses (Lin et al., 2008).

The glutamate receptor has become the focus of much work because it is widely believed that glutamate receptors mediate a central role in learning and memory (Bliss & Collingridge, 1993; Hollmann & Heinemann, 1994; Malenka & Bear, 2004). At glutamatergic synapses, presynaptic terminals contain glutamate-filled synaptic vesicles clustered at the active zone (Figure 1.2b). On the opposite side, the postsynaptic density (PSD) (Fig.1.2b) is the locus for clustering glutamate receptors. Two classes of glutamate receptors, ionotropic receptors (ligand gated ion channels) and metabotropic receptor (G-protein coupled receptors) are clustered together on the postsynaptic membrane at mature
synapses. Metabotropic GluRs (mGluRs) mediate transmembrane signal transduction via trimeric G-proteins. mGluRs are GTP binding protein coupled receptors that induce slow excitatory postsynaptic potentials (EPSPs) and modulate excitatory synaptic transmission via second messenger signalling pathways (Abe et al., 1992).

Figure 1-2 Glutamatergic presynaptic terminals contain glutamate-filled synaptic vesicles clustered at the active zone

(a) Classic drawing of the hippocampal formation by Santiago Ramon y Cajal, and (b) Electron micrograph of an excitatory synapse highlighting the glutamate-filled synaptic vesicles at the active zone of a CA3 presynaptic terminal (arrow) and the postsynaptic density (black arrowheads) on a CA1 dendritic spine (Elias & Nicoll, 2007).

Ionotrophic glutamate receptors (iGluRs) harbor an intrinsic neurotransmitter gated cation channel. iGluRs are ion channels which evoke fast EPSPs (Molnar et al., 2002). There are three different types of iGluRs: AMPA, kainate, and NMDA subtypes (Hollmann, 1999; Burnashev & Rozov, 2000).

1.3.1. AMPA receptors

AMPA receptors are composed from combinations of four subunit types, GluR1, GluR2, GluR3 and GluR4. These subtypes of AMPARs are of a similar size, about 900 amino acids, and share 68-73% amino acid sequence identity (Hollmann, 1999; Burnashev & Rozov, 2000). In the adult hippocampus, two forms of AMPARs are thought to predominate: GluR1/GluR2 heteromers and GluR2/GluR3 heteromers (Citri & Malenka, 2008). The subunit composition determines channel conductance properties and gating kinetics, and also regulates vesicular traffic to and from synaptic sites, therefore critical for
synaptic function and plasticity (Greger et al., 2007). All AMPAR channels are selectively permeable to various cations including K⁺, Na⁺ and Mg²⁺. Normally AMPARs are impermeable to Ca²⁺. GluR2 may serve an important function in regulating this lack of Ca²⁺-permeability of AMPA receptors in vivo, during development as well as during certain abnormal conditions such as might occur in neurodegenerative disorders (Hollmann & Heinemann, 1994).

AMPARs respond instantly to presynaptic glutamate release and mediate fast excitatory neurotransmission. During the first two postnatal weeks in rats, synapses progressively acquire functional AMPARs, which coincide with the maturation of the associated circuits (Durand et al., 1996). At resting membrane potentials, the activation of AMPARs will produce most of the inward currents at excitatory synapses. GluR1 and GluR3 show strong inward rectification, whereas GluR2 displays slightly outward rectification (Boulter et al., 1990; Nakanishi et al., 1990; Verdoorn et al., 1991). Synaptic AMPAR number is vital to determine the amplitude of AMPAR-mediated currents. The number of AMPARs per synapse varies markedly, ranging from about 5 to 200 (Chua et al., 2010).

GluR1 is 96-97% identical at the amino acid level between mammals; the rat, mouse and human (Hollmann & Heinemann, 1994). GluR1 is enriched in synaptic membranes, particularly in the postsynaptic density in the hippocampus. GluR1 can be regulated by MAGUKs and transmembrane AMPAR regulatory proteins (TARPs). GluR1 has four phosphorylation sites, serine 818 (S818), serine 831(S831), threonine 840 (T840) and serine 845(S845) (Banke et al., 2000; Boehm et al., 2006b; Derkach et al., 1999). Phosphorylation of GluR1 can regulate receptor localization, open probability and conductance. The C-terminal domains of GluR1 interact with intracellular signalling proteins and contain phosphorylation sites for protein kinase A (PKA), protein kinase C (PKC), and calcium/calmodulin-dependent protein kinase II (CaMKII) (Braithwaite et al., 2000; Scannevin & Huganir, 2000). Phosphorylation of GluR1 during LTP expression will be described in section 1.4.1.2.

Selective antagonists of AMPARs are 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX) and 6-cyano-7-nitroquininoxaline-2,3-dione (CNQX). Antagonists at AMPA receptors might be powerful neuroprotective agents. GYKI 53784 is a selective and non-competitive AMPAR antagonist. Compared to NMDA receptor antagonists, which normally cause severe side effects such as the impairment of memory functions, GYKI 53784 may have potential as a neuroprotective agent for stroke, trauma and epilepsy (Ruel et al., 2002).
1.3.2. NMDA receptors

The subunits of NMDARs include NR1, NR2 and NR3. There are eight different NR1 subunits generated by alternative splicing from a single gene, four different NR2 subunits including NR2A, NR2B, NR2C, NR2D and two NR3 subunits including NR3A and NR3B (Dingledine et al., 1999; Paoletti & Neyton, 2007). Functional NMDA receptors require the co-expression of at least one NR1 and one NR2 subtype (Hollmann & Heinemann, 1994). The binding sites of NMDARs are important for interaction with other synaptic regulators. Glutamate, glycine, Mg$^{2+}$, Zn$^{2+}$ and a polyamine recognition site are found in NMDARs. Among them, glutamate, glycine and Mg$^{2+}$ are crucial for NMDAR activation. At resting membrane potentials, Mg$^{2+}$ blocks the NMDAR channel pore and makes NMDARs inactive, therefore NMDARs contribute little to the postsynaptic response during basal synaptic activity. Only when postsynaptic depolarization and Mg$^{2+}$ blockade is relieved simultaneously, are NMDA receptor able to conduct currents. Glycine is a co-agonist of NMDARs, and can enhance NMDAR mediated synaptic potentials (Thomson et al., 1989), but only after partial depolarisation of the postsynaptic membrane to relieve the Mg$^{2+}$ block.

NMDARs conduct primarily Ca$^{2+}$ ions, which in turn can trigger intracellular signalling cascades. Although only 5–10% of the total amount of calcium influx comes through NMDA receptors, it is widely accepted that this amount plays a crucial role in synaptic plasticity (Wang et al., 2002; Dur-e-Ahmad et al., 2011). These Ca$^{2+}$ signals can determine the type of synaptic plasticity induced, e.g. LTP or LTD (Amici et al., 2009). Ca$^{2+}$ influx > 5µM induces LTP and modest (<1 µM) postsynaptic Ca$^{2+}$ induce LTD (Bear & Malenka, 1994).

Thus, through NMDA receptors, Ca$^{2+}$ can determine LTP or LTD induced by bidirectional regulation of synaptic plasticity. The unique properties of the NMDA receptor play a key role in the cellular mechanisms thought to underlie learning and memory by defining the receptor as a ‘coincidence detector’ to initiate synapse plasticity and leading to the formation of new neural networks (Dingledine et al., 1999; Genoux & Montgomery, 2007).

Exocytosis of NMDA receptors is tightly related to enhanced NMDAR number and LTP (Nong et al., 2004; Lau & Zukin, 2007). Following synthesis in the endoplasmic reticulum (ER) and further maturation in the Golgi complex, NMDARs are delivered to the cell surface through a process involving exocytosis. Opposite to exocytosis, NMDARs undergo clathrin-mediated endocytosis and after internalization the receptors are sorted to endosomes (Nong et al., 2004). Exocytosis and endocytosis of NMDARs will be described further in section 1.6.5. In addition to regulating synaptic plasticity, NMDARs are involved in the processes of neurological diseases and brain disorders. Excessive Ca$^{2+}$ influx through over stimuli or over activation of NMDARs activates pathological pathways contributing to excitotoxic neuronal cell death. NMDARs are involved in acute and chronic neurological
diseases and disorders including stroke, epilepsy, brain trauma, Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS) and autism (Chen & Lipton, 2006; Ghanizadeh, 2010). For example, Neurotensin, a neuropeptide interacting with the dopaminergic system, is known to intensify neuronal NMDA-mediated glutamate signalling, which may cause apoptosis in autism (Ghanizadeh, 2010).

1.4. LTP and LTD

LTP and LTD at glutamatergic synapses in the hippocampus have been investigated for almost 40 years. In the central nervous system, activity-dependent bidirectional control of synaptic efficacy, as exemplified by various forms of LTP and LTD, is thought to contribute to many forms of experience-dependent plasticity, including learning and memory (Rebola et al., 2010). A long-lasting change in the pattern of synaptic weights would underlie the formation of new information as Santiago Ramony Cajal pointed out 100 years ago. Donald Hebb suggested that memories are acquired through changes in synaptic strength where presynaptic activity correlates with postsynaptic firing (Hebb, 1949). The most extensively studied and therefore prototypic forms of synaptic plasticity are the LTP and LTD observed in the CA1 region of the hippocampus, which is triggered by activation of NMDARs (Malenka & Bear, 2004; Citri & Malenka, 2008).

1.4.1. NMDAR-Dependent LTP

To easily understand the mechanisms of LTP, investigators describe LTP in three phases: induction, expression and maintenance. Induction is the first phase of LTP activated by a brief signal stimulation, and the expression is the biochemical changes resulting in the change in synaptic strength. The maintenance of LTP is the long-lasting cellular changes through activation of maintenance molecules and signalling (Sweatt, 1999).

1.4.1.1. LTP induction

LTP is induced by high-frequency axonal tetanic stimulation (HFS, e.g. 1 sec/100 Hz stimuli) (Cooke & Bliss, 2006) or elicited by a pairing protocol in which low-frequency afferent stimulation is paired with postsynaptic depolarization (Montgomery et al., 2001). Through stimulation of NMDA receptors, chemical LTP (cLTP) can also be induced by brief (3 min) elevation of the concentration of the coagonist glycine in the perfusion solution to a suprasaturating level (100 or 200 µM). The potential activation of glycine receptors is
avoided by including strychnine in the solution (Lu et al., 2001; Sharma et al., 2006). Action potentials or axonal stimulation triggers glutamate release, and glutamate binds AMPARs in the PSD to induce the influx of sodium ions into the postsynaptic neuron, triggering excitatory postsynaptic potentials (EPSP). As a result of EPSP summation, sufficient depolarization over threshold is caused by repeated stimuli with high frequency. Sufficient depolarization will relieve the blockade of Mg$^{2+}$ within the NMDARs which will then allow Ca$^{2+}$ to flow into the neuron. The influx of Ca$^{2+}$ through the NMDAR channel and a consequent rise in Ca$^{2+}$ concentration within the dendritic spine is an absolutely necessary trigger for NMDAR-dependent LTP (Malenka & Bear, 2004). The rapid increase in intracellular Ca$^{2+}$ concentration triggers the LTP induction related enzymes including CaMKII, PKC and PKA (Sweatt, 1999).

1.4.1.2. LTP Expression

Recruitment and insertion of AMPARs into the synapse is vital for LTP expression. In the absence of neural activity, AMPARs can enter synapses without changing the magnitude of synaptic transmission (Kakegawa et al., 2004; Kessels & Malinow, 2009), suggesting a mechanism for a one-for-one exchange of receptors from extrasynaptic to synaptic sites (Makino & Malinow, 2009). During LTP, AMPARs interact with ‘slot proteins’ and are trapped within the postsynaptic density (Kim & Sheng, 2004; Montgomery et al., 2004). At CA1 synapses, LTP is mainly expressed postsynaptically as an alteration in the AMPAR numbers and properties. A possible presynaptic locus of LTP, an alteration in the release probability of transmitter, was a major controversy in the past. A presynaptic expression mechanism requires the release of retrograde messengers to act on presynaptic terminals (Bredt & Nicoll, 2003). It is now believed that the major mechanism of expression of LTP at hippocampal CA1 synapses involves an increase in the numbers of AMPARs within the postsynaptic density, driven through activity dependent changes in AMPAR trafficking (Montgomery et al., 2001; Bredt & Nicoll, 2003; Citri & Malenka, 2008). A major contribution to end the controversy for presynaptic or postsynaptic mechanism was the proposal of the “silent” synapse hypothesis and the evidence supporting it (Montgomery et al., 2001; Bredt & Nicoll, 2003; Citri & Malenka, 2008). Some glutamatergic synapses lack surface-expressed postsynaptic AMPA receptors but contain NMDARs. These excitatory synapses are thought to be postsynaptically "silent " (Montgomery et al., 2001; Malenka & Bear, 2004; Citri & Malenka, 2008), because NMDARs contribute little to basal transmission at resting membrane potentials. Anatomical evidence for silent synapses came from a study using GluR1 immunocytochemistry and showed a proportion of pyramidal neuron spines lacked surface-expressed AMPARs, even though intracellular
AMPARs were detected within the same spine (Molnar et al., 1993; Richmond et al., 1996; Isaac, 2003). LTP has been shown to awaken silent synapses through new AMPARs being rapidly inserted within the postsynaptic density (Montgomery et al., 2001; Malenka & Bear, 2004). GluR1 or GluR4, but not GluR2 and GluR3 are directly inserted into silent synapses during LTP expression (Isaac, 2003). The insertion of GluR1-containing AMPARs into synapses is slow under basal conditions and is strongly stimulated by NMDAR activation (Citri & Malenka, 2008).

It is believed that the PKA, PKC and CaMKII phosphorylation sites on GluR1 might be important for LTP (Boehm et al., 2006a; Esteban, 2007). Phosphorylation of GluR1 by PKC at serine 818 residue is increased during LTP and is critical for LTP expression (Boehm et al., 2006b). AMPA receptor peak response open probability can be increased by PKA through phosphorylation of GluR1 Ser845. (Banke et al., 2000). CaMKII, the essential kinase involved in LTP, increases AMPAR activity during LTP expression (Hayashi et al., 2000; Elias & Nicoll, 2007) and contributes to LTP by increasing phosphorylation of Ser831 of GluR1. CaMKII can mediate LTP by increasing single-channel conductance of GluR1 (Derkach et al., 1999). Phosphorylation of transmembrane AMPAR regulatory protein (TARP) occurs following CAMKII activation and may also be critical for LTP (Cooke & Bliss, 2006; Tsui & Malenka, 2006).

1.4.1.3. LTP Maintenance

Persistent hippocampal LTP has been recorded in behaving rats for as long as a year, and in brain slices LTP can persist for many hours (Abraham et al., 2002; Blitzer, 2005). The maintenance of LTP beyond 1 to 3 hours requires new gene transcription and stable protein synthesis, whereas early LTP (less than one hour) does not require protein synthesis (Barco et al., 2002; Blitzer, 2005). CaMKII, PKC, mitogen-activated protein kinase (MAPK), and cAMP response element binding protein (CREB) are involved in LTP-related protein synthesis. For example, persistent phosphorylation by the atypical PKC isoform, protein kinase Mzeta (PKMz), has been found to maintain late LTP in vitro and in vivo (Pastalkova et al., 2006). However, the identities of the LTP-related proteins remain largely unknown (Ling et al., 2002; Lisman et al., 2002; Blitzer, 2005). Most investigators believe that the incorporation of AMPARs into the PSD is the very important change because it appears to be accompanied by structural changes in the dendritic spines and synapses themselves, an attractive mechanism for maintaining LTP (Shi et al., 2001; Abraham & Williams, 2003; Park et al., 2004).
1.4.2. LTD

LTD is the long lasting decrease of synaptic strength. The persistence of LTD may allow acquisition of new information by restricting the body of previously stored information and suppressing interference (Mallaret et al., 2010).

1.4.2.1. NMDAR-Dependent LTD

LTD can be induced by repetitive low frequency (1-3 Hz) stimulation of CA1 pyramidal cells. NMDAR-dependent LTD was first found at excitatory synapses on hippocampal CA1 pyramidal neurons and shown as a decrease in the excitatory postsynaptic potential which persisted without signs of recovery for more than one hour (Dudek & Bear, 1992; Mulkey & Malenka, 1992). The effects of the induction stimulation could be prevented by application of NMDA receptor antagonists (Dudek & Bear, 1992; Mulkey & Malenka, 1992). Chemical LTD (cLTD) can be induced by a brief application of NMDA (20 µM, 3 min) to hippocampal slices (termed chemLTD), and this correlates with a persistent dephosphorylation of GluR1 on Ser-845 (Kameyama et al., 1998; Lee et al., 1998). Chemically induced LTD (chemLTD) is induced postsynaptically, saturable, sensitive to postnatal age, reversible, and not associated with a change in paired pulse facilitation. ChemLTD and homosynaptic LTD are mutually occluding, suggesting a common expression mechanism (Lee et al., 1998; Sturgill et al., 2009).

Calcium is vital for triggering NMDAR-dependent LTD, as loading CA1 cells with the Ca\(^{2+}\) chelator BAPTA prevented LTD (Dudek & Bear, 1992). In NMDAR-dependent LTD, incoming Ca\(^{2+}\) interacts with calmodulin to activate PP2B (protein phosphatase 2B, also called calcineurin); PP2B dephosphorylates inhibitor -1 and then activates PP1 (protein phosphatase 1). PP1 then dephosphorylates GluR1 leading to LTD (Mulkey et al., 1993; Collingridge et al., 2010).

LTD is mediated by persistent postsynaptic changes, however, there is evidence that NMDAR-LTD may also involve a reduction in the probability of glutamate release and this could be triggered by changes in the presynaptic terminal or by postsynaptic changes that are communicated across the synapse via a retrograde messenger (Stanton, 2003; Collingridge et al., 2010). It is widely believed that the removal of AMPARs via endocytosis from the postsynaptic membrane is the major mechanism of LTD (Malenka & Bear, 2004; Genoux & Montgomery, 2007; Citri & Malenka, 2008).

Hippocampal neurons lacking both GluR2 and GluR3 subunits display normal LTD, suggesting that GluR1 removal contributes to synaptic depression (Meng et al., 2003). During LTD, GluR1 phosphorylation at T840 is regulated by NMDA receptor activation (Delgado et al., 2007). Dephosphorylation of GluR1 at S845 or S831 is associated with the
removal of GluR1 from synapses (Makino & Malinow, 2009). LTD requires PKC phosphorylation of GluR2, therefore, both GluR1- and GluR2-containing receptors seem to participate in the synaptic trafficking associated with LTD (Esteban, 2007).

During LTD, dynamin-dependent NMDAR endocytosis depends on synaptic state. That is, NMDA receptors are resistant to activity-induced changes at silent and recently-silent synapses, and fully 'plastic' after synaptic transition to the active state (Montgomery et al., 2005). NMDAR endocytosis during LTD has been observed in some reports; for example, stimulation of the glycine site of NMDARs initiates signalling through the NMDAR complex, priming the receptors for clathrin-dependent endocytosis and a principal consequence of NMDAR internalization is that the receptors are removed from the cell surface, targeted for degradation or engaged by intracellular biochemical cascades (Nong et al., 2003); Tyrosine dephosphorylation can downregulate NMDA receptor activity and this endocytosis results in decreasing the number of functional channels (Visse et al., 2001). Therefore, NMDARs are not static in the postsynaptic membrane, but are as dynamic as AMPARs with regard to synaptic depression (Montgomery et al., 2005).

1.5. Postsynaptic density (PSD) and MAGUKs

Ultrastructural studies of excitatory synapses have revealed an electron-dense thickening in the postsynaptic membrane which is termed the postsynaptic density (PSD). Over 1,000 proteins (1,461 proteins), involving 133 neurological and psychiatric diseases, have recently been identified in human postsynaptic density (hPSD) (Bayes et al., 2011). A high concentration of glutamate receptors, associated signalling proteins, and cytoskeletal elements are all assembled by a variety of synaptic scaffold proteins into the PSD (Sheng & Hoogenraad, 2007). The PSD stabilizes AMPA receptors (AMPARs) and NMDA receptors (NMDARs) opposed to presynaptic terminals, providing a foundation for synaptic transmission and bidirectional receptor trafficking during synaptic plasticity (Howard et al., 2010). The most important and attractive scaffolding proteins in the PSD are the membrane-associated guanylate kinase homologs (MAGUKs) family which have merged as central organizers of multicomponent protein signaling complexes (Montgomery et al., 2004). The MAGUK family includes SAP97, PSD-95 (SAP90), PSD-93 (Chapsyn 110) and SAP102. MAGUKs share a common domain organization with three N-terminal PDZ domains, a Src-homology 3 (SH3) domain, and a carboxy terminal catalytically inactive guanylate kinase (GK) domain (Elias et al., 2006).
1.5.1. PSD-95

Postsynaptic density-95 (PSD95) is a 95 kDa scaffolding molecule in the brain that clusters postsynaptic proteins including ion channels, receptors, enzymes and other signaling partners required for normal cognition (Joseph et al., 2011). PSD-95 is the principal MAGUK in the hippocampus and has an important role in controlling the levels of AMPARs and NMDARs at glutamatergic synapses (Schluter et al., 2006). Overexpression of PSD-95 increases synaptic strength, enhances synaptic AMPAR number and occludes LTP (Schnell et al., 2002; Nakagawa et al., 2004; Elias et al., 2006). PSD95 does not bind directly to AMPARs, it binds to AMPARs through stargazin and the TARP family. PSD95 binds via a PKA-dependent interaction between the C-terminal tail of stargazin and the first two PDZ domains of PSD95 (Chetkovich et al., 2002; Schnell et al., 2002; Genoux & Montgomery, 2007). Directing interactions between PSD95 and stargazin control synaptic AMPA receptor number, whereas knockdown of PSD-95 decreases surface expression of AMPARs (Schluter et al., 2006; Ehrlich et al., 2007).

PSD95 can interact with NMDARs (Petralia et al., 2009) and interacts with both NR2A and NR2B (Hoe et al., 2006). The interaction is mediated by binding of the C terminus of the NMDA receptor subunits to the first two PDZ domains of PSD-95, particularly NR2B binding to PDZ2 domain whereas NR2A binds to both PDZ1 & 2 domains (Niethammer et al., 1996). Synaptic localization and association with PSD-95 increases stability of hippocampal neuronal NMDARs (Hoe et al., 2006). PSD95 participates in the switch of NR2B-NMDARs to NR2A-NMDARs during early development (Elias et al., 2008). Voltage-gated, Shaker-type, K(+) (K(V)1) channels are key binding partners of PSD95 in postsynaptic membrane as loss of PSD95 induced a strong constriction associated with a loss of functional K(V)1 channels (Joseph et al., 2011).

1.5.2. SAP102

SAP102 is extensively expressed in dendrites, axons, the postsynaptic density and cytoplasm in both young and mature neurons (Zanni et al., 2010; Zheng et al., 2010). High expression of SAP102 exists in the first postnatal week but decreases after development (Zheng et al., 2011). The majority of SAP102 is clustered at the PSD and stabilized through interactions involving its SH3/GK domains (Zheng et al., 2010). SAP102 mediates synaptic trafficking of AMPA and NMDA receptors during synaptogenesis and can mediate synaptic trafficking of NR2B- and NR2A-NMDARs during neurodevelopment (Sans et al., 2000; Elias et al., 2008). Overexpression of SAP102 increases AMPAR and NMDAR EPSC amplitudes, whereas knockdown of SAP102 reduces both AMPAR and NMDAR EPSC amplitudes (Elias et al., 2008). SAP102 is also known to be required for the induction of
LTP and spike-timing-dependent plasticity (Cuthbert et al., 2007). SAP102 mutant mice showed a specific spatial learning deficit and SAP102 function may be directly relevant to the mechanisms of autism because MAGUK proteins in the NMDA receptor complex bind directly to neuroligin, an autism susceptibility gene (Irie et al., 1997; Yan et al., 2004; Cuthbert et al., 2007).

1.5.3. PSD-93

PSD-93 directly binds to NMDA receptors and indirectly to AMPA receptors via transmembrane AMPA receptor regulator proteins (Schnell et al., 2002; DeGiorgis et al., 2006). The first and second PDZ domains of PSD-93 bind to the C-termini of NR2A and NR2B as well as to neuronal nitric oxide synthase (nNOS) (Roche et al., 2001; Zhang et al., 2010). NMDAR-triggered neurotoxicity is related to excessive Ca\(^{2+}\) loading and an increase in nitric oxide (NO) concentration, and PSD-93 deficiency could block NMDAR-triggered neurotoxicity by disrupting the NMDAR-Ca\(^{2+}\)-NO signaling pathway and reducing expression of synaptic NR2A and NR2B (Zhang et al., 2010). Deletion of PDZ domains of PSD-93 not only disrupted the interaction between PSD-93 and NR2A or NR2B but also reduced NMDAR clustering at cell membranes in vitro (Kim et al., 1996; Zhang et al., 2010).

The amino acid sequence of PSD-93 and PSD-95 reveals 71% identity, with each protein having the same domain organization including the palmitoylation motif (Delint-Ramirez et al., 2010). PSD-93 and its relative PSD95 exist as disulfide linked complexes in rat brain, consistent with head-to-head multimerization of these proteins in vivo and this interaction could form a multimeric scaffold for the clustering of receptors, ion channels, and associated signalling proteins (Hsueh et al., 1997). Although PSD93 & PSD95 have nearly identical distributions at synapses, evenly distributed throughout the PSD, PSD-93 mutant mice exhibit deficits in LTP and normal LTD, whereas the facilitation of LTP induction and disruption of LTD were observed in PSD-95 mutant mice. This may be because these MAGUKs form distinct NMDA receptor signalling complexes that differentially regulate the induction of LTP by different patterns of synaptic activity (DeGiorgis et al., 2007; Carlisle et al., 2008).

1.6. SAP97

SAP97 is the rat homologue of the Drosophila (Dlg) and human (hDlg) discs large tumor suppressor protein. SAP97 contains the nucleotide and deduced amino acid sequence of a 97 KDa protein. SAP97 is widely expressed in brain and multiple organs,
especially in epithelial cells in the body. SAP97 is enriched in excitatory synapses, including the PSD and presynaptic sites. SAP97 is strongly implicated in trafficking of NMDA and AMPA receptors in synapses, may participate in synaptic plasticity and could be involved in many neurological diseases.

1.6.1. The Structure of SAP97

The human homologue of Drosophila Dlg, termed hDlg, and its rat counterpart SAP97, localize at the pre- and postsynaptic membrane sites as well as the membrane of epithelial cells (Lue et al., 1994; Muller et al., 1995; Valtschanoff et al., 2000; KloEcker et al., 2002). Dlg1 (Drosophila tumor suppressor gene d1g-A), Zo-1 (zonula occludens proteins) and Zo-2 are structurally close to SAP97 (Müller et al., 1995). SAP97 is composed of three PDZ (PSD-95, Dlg, and ZO-1) repeats, a single SH3 domain, a HOOK domain (a part of the SH3 domain), and an inactive guanylate kinase (GK) domain. Additionally, SAP97 contains two regions of alternative splicing. One region is the N-terminal region and the other region is located between the SH3 and GUK domain. In the N-terminal region of SAP97 there are two spliced insertions, I1A and I1B, located between the unique N-terminal domain of SAP97 and the first PDZ repeat. The region between the SH3 and GUK domains of SAP97 contains four alternatively spliced insertions, which have been characterised, I2, I3, I4 and I5 (Fig. 1.3) (Lue et al., 1994; McLaughlin et al., 2002).

The alternatively spliced insertions endow SAP97 special functions. For instance, insertion I3 is responsible for localization of SAP97 to the plasma membrane. The I3 insert is recognized by the actin/spectrin-associated protein, 4.1N, and this interaction is responsible for synaptic targeting of SAP97 and AMPA receptors (Rumbaugh et al., 2003; Zheng et al., 2011). As previously reported, the role of the I2 insert is to target SAP97 to the nucleus (Rumbaugh et al., 2003; Robertsa et al., 2007; Yamada et al., 2007). SAP97-I2-containing mRNAs appeared to be less abundant than SAP97-I3-containing mRNAs (Nikandrova et al., 2010). In skin and cervix, I3 variants are found in the cytoplasm. I2 variants are found at the cell periphery of differentiated epidermal and cervical keratinocytes; Nuclear localization of I2 variants was evident in both tissues (Robertsa et al., 2007).
α- and β-isoforms differ in their N termini, with the former containing a palmitoylation motif (cysteine residues C3 and C5) and the latter an L27 domain. Alternatively spliced sequences (I1a, I1b, I2, I3, I4, I5) are indicated between the SH3 and GUK domain (Waites et al., 2009).

Figure 1-3 Schematic domain organization of SAP97.

The N-terminal alternatively spliced region of SAP97 is capable of binding several SH3 domains and also moderates the level of protein oligomerization. The most prevalent hDlg isoform containing 1A, 1B, and I3 can presumably recruit distinct SH3-containing proteins to sites of cell-cell contact, e.g. at synapses (McLaughlin et al., 2002). Alternative mRNA splice variants of SAP97 are differentially distributed in cells. For example, similar to epithelial cells, deleting the SH3 or GUK domains has little effect on the spine localization of SAP97, whereas deleting the hook region (ΔI3) leads to a reduced efficacy of targeting SAP97 to the spine head (Rumbaugh et al., 2003). In myocardia cells co-expressing I3, green fluorescent protein-tagged Kv1.5 channels were organized in plaque-like structures at the plasma membrane, whereas intracellular aggregates of channels predominated with the I2 isoform (Godreau et al., 2003).

Due to existence of many different alternatively spliced insertions, the roles and functions of SAP97 are quite complicated. In addition to many insertions, SAP97 contains alternative N termini expressing either double cysteines (Figure1-3) that normally are palmitoylated (α-isoforms) or an L27 domain (β-isoforms) (Schluter et al., 2006; Waites et al., 2009). The unique N-terminal domains confer distinct subsynaptic localizations onto SAP97, targeting the palmitoylated α-isofom to the postsynaptic density (PSD) and the L27 domain-containing β-isofom primarily to non-PSD, perisynaptic regions. βSAP97 has a longer N-terminal region featuring the L27 domain and is the most prominent in the brain (Schluter et al., 2006; Mauceri et al., 2007). In previous work using hippocampal slice cultures, overexpression of βSAP97 produced either no effect on AMPAR EPSCs or an increase, which was much less than that observed with overexpression of PSD-95 (Nakagawa et al., 2004; Schluter et al., 2006; Howard et al., 2010). Consistent with these findings, overexpression of βSAP97 containing the I3 insert, which is required for synaptic targeting, had no significant effect on either AMPAR EPSCs or NMDAR EPSCs (Schnell et al., 2002; Rumbaugh et al., 2003; Ehrlich & Malinow, 2004; Nakagawa et al., 2004;
Moreover, the function of the L27-containing βSAP97 isoforms was activity dependent and completely masked by PSD-95α, the principle endogenous isoform of PSD-95. In contrast, the cysteine doublet-containing, palmitoylated α isoforms SAP97 increased AMPAR EPSCs independent of the level of both endogenous PSD-95 and activity (Schluter et al., 2006).

1.6.2. SAP97 Binding partners

SAP97 has been shown to interact with a number of binding partners including cytosolic structural proteins. The first identified binding partner of SAP97 was protein 4.1 (Lue et al., 1994; McLaughlin et al., 2002). Protein 4.1 is concentrated at cell to cell junctions and in the nucleus where it is important for both structural stability and signal transduction. This function of protein 4.1 is important for SAP97 anchoring. Protein 4.1 interacts with the HOOK domains of SAP97 and deletion of the HOOK domain from SAP97 prevents its association with the plasma membrane (Lue et al., 1994; Marfatia et al., 1995; Hoover & Bryant, 2000). The hook domain is a sequence situated between the SH3 and GK domains of Dlg and is analogous to the I3 region of SAP97.

The N terminus of SAP97 plays an important role in the selection of binding partners and the localization of SAP97 at adhesion sites, as well as the clustering of ion channels in heterologous cells (Wu et al., 2002). For example, the N terminus of SAP97 has a myosin VI binding site. Direct binding between myosin VI, SAP97 and the AMPA receptor subunit, GluR1, may be involved in trafficking AMPA receptors to and from the postsynaptic plasma membrane (Wu et al., 2002). SAP97 and PSD95 PDZ domains can directly interact with KIF1Bα (kinesin family member 1Bα), and S-SCAM (synaptic scaffolding molecule). SAP97 is widely distributed in both dendrites and axons along with KIF1Bα and S-SCAM, and SAP97 biochemically associates with KIF1Bα and S-SCAM, and these results strongly suggest that KIF1Bα plays an important role in the axonal transport of a variety of SAP97- and S-SCAM-associated cargos, such as Kv1.4 potassium channel complexes (Mok et al., 2002; Kim & Sheng, 2004). In addition to binding KIF1B, SAP97 can also bind, through its GK (guanylate kinase-like) domain, to KIF13B/GAKIN (kinesin family member 13B) (Kim & Sheng, 2004). The binding of GKAP (GUK-associated protein) to the GUK domain of SAP97 is regulated by a series of intramolecular interactions between the SH3 and GUK domains (Wu et al., 2000). A unique feature of this interaction is the unusual location of the SAP97 binding region which could provide insights into the mechanism of dynamic regulation of the cell-cell contact structure in normal and disease states (Asaba et al., 2002; Kim & Sheng, 2004).

SAP97 and PSD-95 also interact with A-kinase-anchoring protein 79/150 (AKAP79/150) (Colledge et al., 2000), a scaffold for PKA, PKC and the Ca^{2+}/calmodulin
dependent protein phosphatase calcineurin (also known as PP2B). The interaction might bring these kinases and phosphatases close to their specific substrates in the synapse. For instance, the SAP97–AKAP complex facilitates the phosphorylation by PKA of the glutamate receptor GluR1 (Colledge et al., 2000; Kim & Sheng, 2004). Therefore the SAP97–AKAP79 complex might be important for the recruitment of kinases and phosphatases to synaptic AMPARs. In contrast, phosphorylation by CaMKII of SAP97 in the N-terminal L27 domain promotes synaptic targeting of SAP97, and of its binding partner GluR1 (Mauceri et al., 2004). The L27 domain is involved in SAP97 heteromultimerization and homomultimerization with itself and other L27 domain-containing proteins, such as calmodulin-dependent serine protein kinase (CASK) (Lee et al., 2002). CASK requires a L27 domain–mediated interaction to associate with the SAP97/NMDAR complex in hippocampal neurons (Jeyifous et al., 2009). Thus, L27 domains appear to play a central role in the higher order organization of multi-scaffold assemblies (Petrosky et al., 2005).

1.6.3. Functions of SAP97

SAP97 has many important functions in the brain and other organs including skin, kidney, and heart. Absent or mutant SAP97 can result in severe deficiency and diseases. For example, the loss of dlg function in Drosophila disrupts cellular growth control, apicobasal polarity, and cell adhesion of imaginal disc epithelial cells, resulting in embryonic lethality (Caruana & Bernstein, 2001). SAP97 lacking SH3 and GK domains demonstrate an essential role of the SH3 and GK domains for craniofacial and palatal development. Mice with mutant SAP97 lacking the SH3 and GK domains have a cleft secondary palate, and die perinatally (Caruana & Bernstein, 2001; KloEcker et al., 2002). Dlgh1−/− mice developed severe urinary tract abnormalities, including congenital hydronephrosis, which is the leading cause of renal failure in infants and children (Zhen et al., 2006).

SAP97 participates in the regulation of cardiac delayed rectifier currents. The Kv1.5 channel is a member of the superfamily of voltage dependent K+ channels. The Kv1.5 channel is expressed in mouse ventricle and human atrium. It carries the ultra-rapid component of the delayed-rectifier K+ channel current in cardiomyocytes (Nattel et al., 1999). In addition to regulating K+ channels, SAP97 is implicated in regulating the pools of cardiac sodium channels Nav1.5 in cardiomyocytes. The cardiac sodium channel Nav1.5 initiates the cardiac action potential, thus playing a key role in cardiac excitability and impulse propagation (Petitprez et al., 2011).

Overexpression of SAP97 at excitatory synapses, enhances AMPAR and NMDAR currents (Howard et al., 2010), spine enlargement, and increases in miniature EPSC (mEPSC) frequency, indicating that SAP97 has both postsynaptic and presynaptic effects.
on synaptic transmission (Rumbaugh et al., 2003). SAP97 overexpression also changes the paired-pulse ratio (PPR), indicating a change in presynaptic release probability through trans-synaptic signalling (Howard et al., 2010). However, neurons cultured from SAP97 mutant embryos develop normally and form synapses with normal levels of AMPARs and no detectable abnormalities (KloEcker et al., 2002; Schluter et al., 2006). Mice harboring a truncation of SAP97 do not survive as neonates, because craniofacial deformities prevent feeding (Caruana & Bernstein, 2001), however cultured dissociated neurons from these embryos exhibit normal glutamatergic synaptic transmission (KloEcker et al., 2002; Howard et al., 2010). These findings raise the possibility that Dlg-MAGUK family members share redundant functions in neurons and loss of one can be partially compensated by other family members (Schluter et al., 2006). Due to ill-defined spatial and temporal distributions, it is controversial how SAP97 scaffolds & regulates AMPARs at the postsynaptic membrane. Considerable work needs to be done to examine how SAP97 isoforms regulate the levels, dynamics, and subsynaptic localization of AMPARs and NMDARs and other transmembrane receptors in neurons.

1.6.4. SAP97 and AMPARs

PSD-MAGUKs generally interact with AMPARs through AMPAR auxiliary subunits known as transmembrane AMPAR regulatory proteins (TARPs), which have PDZ-binding motifs (Chen et al., 2000; Howard et al., 2010). SAP97 is the only member of the MAGUK family to interact directly with the AMPAR subunit GluR1. Synaptic plasticity including LTP and LTD require the trafficking and synaptic insertion of GluR1. Because the recruitment of GluR1 is a critical step for inducing and maintaining LTP, the interaction between GluR1 and SAP97 is one of most interesting topics for many investigators to study MAGUKs in regulating AMPARs in synaptic plasticity. SAP97 directs GluR1 forward trafficking from the Golgi network to the plasma membrane. The minus-end directed actin-based motor, myosin VI and SAP97 form a trimeric complex with GluR1 and it is thought that SAP97 may serve as a molecular link between GluR1 and the actin-dependent motor protein myosin VI carrying AMPA receptors to and from the postsynaptic plasma membrane (Wu et al., 2002).

The role of SAP97 in the trafficking and synaptic location of AMPARs is far from solved with multiple labs reporting different data (Caruana & Bernstein, 2001; Howard et al., 2010). Some investigators found that overexpression of SAP97 enhanced the amplitude of AMPAR-mediated mEPSCs (Nakagawa et al., 2004). SAP97 overexpression also rescues AMPAR currents reduced by RNAi-mediated knockdown of PSD-95 (Schluter et al., 2006). However, other investigators indicated that overexpression of SAP97 did not change AMPA currents and loss of SAP97 did not change AMPA EPSCs either (KloEcker et al., 2002; Schnell et al., 2002; Ehrlich & Malinow, 2004; Schluter et al., 2006). Like other
PSD-MAGUKs, SAP97 may be sufficient, but not individually necessary, for AMPAR trafficking to synapses (Regalado et al., 2006; Howard et al., 2010). One study showed that SAP97 only interacts with GluR1 during its forward trafficking to the plasma membrane, suggesting that SAP97 acts on GluR1 solely before its synaptic insertion and that it does not play a major role in anchoring AMPARs at synapses (Sans et al., 2001). Whether SAP97 is a “slot protein” and “docking site” in the PSD to lock AMPA receptor or whether it only interacts with GluR1 before its synaptic insertion is uncertain. SAP97 interacting with other AMPARs subunits is also controversial. One report showed that SAP97 was associated with GluR1 but not GluR2 or GluR3 (Leonard et al., 1998). However, another report revealed that SAP97 antibodies immunoprecipitated GluR2 and GluR1 but almost no GluR3 and a small amount of GluR4 (Hall & Soderling, 1997; Sans et al., 2001). These conflicting data may be due to the multiple alternatively spliced SAP97 isoforms and their poorly-defined spatial and temporal distributions (Rumbaugh et al., 2003; Nakagawa et al., 2004; Schluter et al., 2006; Waites et al., 2009).

1.6.5. SAP97 and NMDARs

SAP97 is also implicated in targeting and trafficking NMDA receptor at synapses. Like other PSD- MAGUKs, SAP97 interacts directly via its PDZ domains with NMDAR PDZ-binding motifs (Sheng & Sala, 2001; Howard et al., 2010). SAP97 plays a role in regulating NMDA receptors through the NR2A/2B subunits (Mauceri et al., 2007; Nikandrova et al., 2010). SAP97 directly associates with the NR2A subunit of the NMDA receptor through its PDZ1 domain in a CaMKII regulated manner. CaMKII phosphorylates SAP97 on two major sites, one located in the N-terminal domain (Ser39) and the other in PDZ1 domain (Ser232). SAP97-Ser39 phosphorylation, triggered by intracellular calcium, releases the SAP97/NR2A complex from the endoplasmic reticulum to drive SAP97 to the postsynaptic compartment (Mauceri et al., 2007). NMDA receptor activation induces CaMKII-dependent phosphorylation of SAP97-Ser-232 to disrupt the NR2A interaction (Gardoni et al., 2003). Thus, CaMKII-dependent phosphorylation of SAP97 controls both NR2A trafficking and insertion at synapses (Mauceri et al., 2007).

In immature neurons, SAP97 prefers interacting with NR2A than NR2B and overexpression of SAP97 at this stage can increase synaptic NMDAR number (Howard et al., 2010). In utero, overexpression of SAP97 can drive the NMDAR subunit switch, resulting in both larger and faster NMDAR currents than normal neurons at the same developmental stage, indicating a potential role for SAP97 in controlling both the number and the specific subunit composition of synaptic NMDARs (Howard et al., 2010). βSAP97 is highly effective at trafficking NMDARs. βSAP97 significantly increases the amplitude of NMDAR-mediated EPSCs but did not alter the paired pulse ratio (PPR), indicating that the
increases in NMDA receptor currents are most likely due to postsynaptic changes (Howard et al., 2010).

Previous work has indicated that SAP97 overexpression has no effect on glutamatergic synaptic transmission in mature neurons (Schluter et al., 2006). βSAP97 overexpression did not enhance NMDAR currents over WT levels in mature neurons, and in loss-of-function experiments, using SAP97 conditional KO mice, glutamatergic synaptic transmission and LTP were normal in mature neurons (Howard et al., 2010). Therefore, according to these studies, overexpression or knockdown of SAP97 is compensated by other PSD-MAGUKs and SAP97 may have more important roles in immature neurons than mature ones.

SAP97 is important for the sorting of NMDARs in hippocampal neurons. A recent study (Jeyifous et al., 2009) showed that there is a novel secretory pathway for NMDAR trafficking in hippocampal neurons and the majority of NMDARs use this pathway. In this novel trafficking pathway, SAP97 and CASK are required for the sorting of NMDARs from AMPARs in the somatic ER, causing a majority of NMDARs to bypass somatic Golgi in favor of dendritic Golgi outposts (Jeyifous et al., 2009). The new trafficking pathway allows NMDA receptors to be more efficiently delivered to synapses and may provide a platform for local control of NMDAR insertion near synapses. When this alternative pathway is unavailable, NMDARs can traffic to synapses via the conventional secretory pathway, as evidenced by the accumulation of NMDARs in somatic Golgi during the knockdown of SAP97 or CASK. When SAP97 was knocked down, endogenous NMDAR localization in dendritic Golgi outposts was lost. Furthermore, the levels of synaptic NMDARs were altered by knockdown of SAP97 or CASK, and found that either reduced synaptic NMDARs by 30–40%. These values are consistent with the 45% decrease in NMDAR EPSCs observed previously with SAP97 knockdown (Nakagawa et al., 2004; Jeyifous et al., 2009). Therefore, SAP97 is involved in NMDAR trafficking and further affects synaptic NMDARs in hippocampal neurons.

1.7. The aims of the study

SAP97 is implicated in AMPAR & NMDARs trafficking from the ER to spines, but there is no direct evidence to reveal whether SAP97 effectively regulates NMDARs at intracellular pools and extra-synaptic pools in synaptic plasticity. Thus, in this research, we have used electrophysiology and other approaches to investigate the functional properties of SAP97 during synaptic plasticity in hippocampal neurons. Why is it important to study the functional role of SAP97 in the brain? As a member of the MAGUK family, SAP97 is a
major scaffolding protein at synapses and is important for regulating glutamate receptors at synapses. SAP97 directly interacts with GluR1 and thus it could be vital for synaptic plasticity. However, due to the germline knockout causing a lethal feeding deficit, and multiple spliced SAP97 isoforms, the roles of SAP97 are poorly understood.

In this study, our specific aims are as follows:

(1) To investigate specific SAP97 isoforms including αSAP97-I3, βSAP97-I3 and βSAP97-I2 individually and study whether they affect the function of AMPARs in hippocampal neurons;

(2) To investigate whether SAP97 isoforms regulate LTP and LTD and underlying synaptic plasticity mechanisms;

(3) To investigate whether SAP97 isoforms affect synaptic and extrasynaptic localization of AMPARs and NMDARs during synaptic plasticity.

Our hypotheses are as follows:

(1) SAP97 isoforms will regulate baseline AMPAR & NMDAR EPSCs in hippocampal neurons;

(2) SAP97 isoforms will affect AMPAR and NMDAR mediated synaptic currents during LTP and LTD expression in hippocampal neurons;

(3) SAP97 isoforms will affect the surface distribution of AMPAR and NMDAR mediated currents during LTP and LTD in hippocampal neurons.
Chapter 2. Methods

The handling of animals and experimental protocols involving animals were approved by the University of Auckland Animal Ethics Committee. In all experiments in this thesis, P0 Wistar rat pups were used. Animals were bred and supplied by the Vernon Jansen Unit, The University of Auckland.

2.1. Primary hippocampal neuron culture

12 mm round glass coverslips were coated with Poly-D-lysine (PDL, BD 354210 or Sigma P1149) to allow the neurons and glial cells to adhere to coverslips. 10 µg/ml PDL was dissolved in sterile phosphate buffered saline (PBS; in mM: 2.68 KCl, 136.89 NaCl, 10.15 NaH₂PO₄ and 1.76 KH₂PO₄) and stored at 4°C. Coverslips were sterilised by soaking in 69% nitric acid overnight and then rinsing with distilled water and stored in 100% ethanol. Coverslips were coated one day before plating. Ethanol was flamed off coverslips and placed into each culture well with 1ml PDL/per well (Falcon 353046). Plates were gently swirled until the poly-D-lysine totally covered the entire surface of the coverslips. Culture plates were incubated overnight at 37°C. Dissecting tools were sterilised for 20 minutes under UV light and stored in 70% ethanol. Postnatal day zero (P0) Wistar rats were killed by decapitation with sharp scissors and the head transferred into a 60mm ice-cold dish. To remove the brain, the skull was cut medially to expose the entire surface of the brain. The brain was then gently scooped out with a spatula blade into ice cold Hanks’s Balanced Salt Solution (HBSS: 9.5 g HBSS (Sigma-Aldrich H2387), 2.38g HEPES (Fluka 54457 or Sigma H3375), pH7.2 with 5M NaOH). To tease the two hemispheres apart, sharp tweezers were used to anchor the brain by the cerebellum and the two hemispheres teased apart with the curved forceps until the midbrain region was exposed and the hippocampus was visible. Hippocampi were cut away from the midbrain and transferred to a new dish with cold HBSS. Fine forceps were used to tease away the meninges. Hippocampi were transferred with a wide fire polished pipette to a new ice cold dish with HBSS and moved to a Class II hood. Hippocampi were enzymatically digested with Papain (Worthington Biochemicals LK003178) in 5 ml of warm HBSS for 15 minutes at 37°C in the incubator or water bath, mixing it at 5 minute intervals. During the incubation time, PDL from the wells of plates was moved and replaced with 2ml per well of sterile room temperature PBS. The Papain was inactivated by the addition of warm serum medium (4.5 ml Minimum Essential Medium with 1x GlutaMax-1 supplement). The serum medium was
then removed and replaced by 5 ml of prewarmed neurobasal media with B27. Hippocampi were dissociated gently by triturating with a fine tipped polished glass pipette until the solution was cloudy and homogenous. The large tissue chunks were allowed to settle before the cell solution was transferred to a new tube. This was again repeated to ensure only dissociated cells were plated. Cells were plated at ~1x10^6 cell/mL with 2 mL per 35 mm culture well. Generally the hippocampi from one pup generated sufficient hippocampal neurons for 1x6 well culture plate. Dissociated cultures were maintained in a 5% CO2 incubator (Sanyo) at 37 °C. To remove the cell debris, the day after plating, 25% (0.5 mL) media was replaced per well with fresh media (500 mL Neurobasal medium (Gibco)+ 10mL B27 supplement (Invitrogen) +5 mL glutamax (Invitrogen). 50% media replacement was repeated weekly until dissociated cells were utilised for experiments. The dissociated cultures were maintained for up to three weeks in vitro.

2.2. Molecular Biology

2.2.1. Expression constructs

βSAP97 containing the I1b, I3, and I5 domains was fused at its C terminus with enhanced green fluorescent protein (EGFP) and subcloned into the XbaI/EcoRI backbone of the lentiviral vector pFUGW (Lois et al.,2002) to generate the plasmid pFU-rSAP97I3-EG (EMBL accession number AM710297; http://www.ebi.ac.uk/EMBL). αSAP97–EGFP and ΔSAP97–EGFP were constructed similarly but have alternative N termini: αSAP97–EGFP starts with the palmitoylation sequence MDCLICIVTTK; ΔSAP97–EGFP lacks this sequence and begins with exon 2. The expression constructs for rat αSAP97–EGFP, αSAP97–EGFP with short hairpin to PSD-95 (sh95), and EGFP–βSAP97 with sh95 were gifts from Robert Malenka (Stanford University, Stanford, CA) (Schluter et al., 2006). Rescue of shRNA mediated knockdown was achieved by coexpressing an shRNA-resistant βSAP97-EGFP and shRNA-SAP97 (S12) in the pFU-rSAP97I3-EG vector. Sequence analysis reveals that αSAP97 has no unique domains: αSAP97 is identical to βSAP97 with the exception of the palmitoylation sequence, and αSAP97 shares this palmitoylation sequence with other MAGUKs (specifically αPSD93). Therefore it was not possible to create an shRNA that specifically targets αSAP97. To knockdown expression specifically of βSAP97, silent mutations in codons encoding N-terminal amino acid residues 6, 7, 8 and 9 of βSAP97-EGFP was achieved by PCR with oligonucleotides containing the following sequence ATG-CCG-GTC-CGG-AAG-CAG-GAC-ACa-CAa-AGA (bold: start codon; lower case: nucleotide changes). Note that this sequence is unique to βSAP97 and present near its L27 domain. The shRNA-SAP97 (S12) under the H1 polymerase III promoters was
subcloned into the BsiW1-Pac1 sites upstream of the ubiquitin promoter/βSAP97-EGFP expression cassette.

2.2.2. Transformation

Competent bacterial DH 5α E. coli cells (stored -80°C, Invitrogen 18265-017) were directly placed on ice to thaw. DH 5α E. cells were gently mixed with a sterile pipette tip and 50µl of cells were removed to a sterile microcentrifuge tube previously cooled on ice. Unused DH 5α E. coli cells were refrozen in a dry ice ethanol bath for 5 minutes before returning to -80°C. 1µl of plasmid containing the gene of interest (e.g. βSAP97-EGFP) was added into 50µl DH5α E coli cells, making a swirling motion to mix without damaging the cells. DH5α E. coli cells were incubated on ice for 20 minutes before undergoing heat shock for 45 seconds to 1 minute at 42°C to lyse the cells and allow DNA uptake. Cells were placed back on ice for 2 minutes to allow the membrane to reform before adding 950µl of Luria-Bertani (LB, Merck 110283) medium (room temperature, no antibiotics) to each tube. Cells were incubated at 37°C for 1 hour at 225 rpm. 50 µl to 100 µl of cells were dispersed with the cell spreader (previously stored in EtOH and flamed) onto an kanamycin (50 µg/mL) containing agar plate. The agar plate was left at room temperature for 5 minutes to absorb the inoculums before inverting and incubating overnight at 37 degrees for 16 hours or until colonies in the agar plates were of the desired size. Single, well-isolated colonies were selected using a sterile small pipette tip the following day to inoculate a starter culture of 2 ml LB containing Kanamycin. Cells were incubated overnight at 37°C with vigorous shaking at 300 rpm and then the DNA was extracted by Mini or Maxi plasmid preparation.

2.2.3. Mini-Plasmid Preparation

The PureLink™ Quick Plasmid Miniprep Kit (Invitrogen K2100-11) was used to isolate high quality plasmid DNA from E. coli cells. 2 mL of cells from the overnight LB culture was placed into a microcentrifuge tube and centrifuged to pellet the cells. All medium was then removed from the cell pellet. The unused overnight culture was placed at 4°C for later Maxi plasmid preparation. Cells were resuspended in 250 µl Resuspension Buffer (R3: 50 mM Tris-HCl, PH8.0; 10mM EDTA). 250 µl Lysis Buffer (L7, 1%SDS, 200 mM NaOH) was then added to lyse the cells and gently inverted 5 times followed by a 5 minute incubation at room temperature. Precipitation Buffer (N4) was added and mixed immediately by inverting the tube until the solution was homogeneous. The mixture was centrifuged at ~12,000 xg for 10 minutes at room temperature, using a microcentrifuge to
separate the lysate from lysis debris. The supernatant of the mixture was then loaded onto a spin column and placed inside a 2 ml wash tube.

To precipitate, wash and elute the DNA, an Invitrogen kit (Invitrogen K2100-10) or self prepared solutions could be used. When using the Invitrogen kit, the spin column with the supernatant was centrifuged at ~12,000 xg for 1 minute and the flow through discarded. 500 µl Wash Buffer (W10) with ethanol was added to the column before incubating for 1 minute at room temperature. The column and tube were centrifuged for 1 minute at ~12,000 xg and the flow-through discarded. The column and tube were washed once more with 700 ul Wash Buffer(W9) and then centrifuged at ~12,000 xg for 1 minute. The flow-through was discarded then the column and wash tube were again centrifuged at ~12,000 xg for 1 minute. DNA was eluted by placing the spin column in a clean 1.5 ml recovery tube and adding 75 µl of TE Buffer (TE, 10mM Tris-HCl, PH8.0, 0.1mM EDTA). The column and recovery tube were incubated for 1 minute at room temperature and centrifuged at ~12,000 xg for 2 minutes. The recovery tube contained purified plasmid DNA and was stored at ~20°C.

When using self prepared solutions, 1 ml of 100% ethanol was added and mixed by inversion to precipitate the DNA. After centrifugation at ~12,000 xg for 10 minutes to pellet the DNA, the supernatant was aspirated. The DNA pellet was washed with 70% ethanol and centrifuged at ~12,000 xg for 5 minutes. The supernatant was aspirated and the DNA pellet was left to dry for ~10 minutes at room temperature. DNA was resuspended in 50µl of TE buffer (10 mM Tris, 0.1 mM EDTA) containing RNase (1:100) by leaving for 30 minutes then vortexing. DNA was then stored at -20°C.

2.2.4. Maxi Plasmid Preparation

PureLink Hipure Plasmid DNA Purification Kit (Invitrogen K2100-27) was used to purify DNA. A high copy number plasmid typically yielded 2-6 µg DNA/ml LB (Luria Bertani) culture. Transformed E.coli cells were grown overnight in LB medium and used in transition between the exponential phase and stationary phase. The PureLink™ Hipure Filter Maxi Columns are prepackaged with the Filtration cartridge inserted into the column housing. 30 ml Equilibration Buffer (EQ1, 0.1 M Sodium acetate, pH 5.0 0.6 M NaCl 0.15% TritonR X-100) was directly applied into the Filtration Cartridge, which was inserted into the Maxi Column, allowing the solution in the HiPure Filter maxi Column to drain by gravity flow. The E. coli cells from a 400 ml overnight culture were pelleted by centrifuging at 4,000 xg for 10 minutes at 4°C. All of the supernatant was removed and Resuspension Buffer (R3, RNase A 20 mg/ml, 50 mM Tris-HCl, pH 8.0 10 mM EDTA) added to the pellet and the cells resuspended until homogeneous. 10 ml Lysis Buffer [L7, 0.2 M NaOH 1% (w/v) SDS]
was added and mixed gently by inverting the capped tube until the lysate mixture was thoroughly homogeneous, following incubation at room temperature no more than 5 minutes. 10ml Precipitation Buffer (N3, 3.1 M Potassium acetate, pH 5.5) was added and mixed immediately by inverting the tube until the mixture was thoroughly homogeneous. The precipitated lysate was transferred into the equilibrated HiPure Filter Maxi Column to allow the lysate to run through the filter by gravity flow (10 -15 minutes). The flow through was discarded. The precipitate was washed with 10ml Wash Buffer (W8, 0.1 M Sodium acetate, pH 5.0, 825 mM NaCl) through the HiPure Filter Maxi Column by gravity flow. Immediately after the HiPure Filter Maxi Column had stopped dripping, the inner Filtration Cartridge was removed from the column. The Maxi column was washed with 50 ml of Wash Buffer (W8) by gravity flow, and the flow-through discarded. A sterile 50 ml centrifuge tube was placed under the HiPure Filter Maxi column and 15ml Elution Buffer (E4, 100 mM Tris-HCl, pH 8.5 1.25 M NaCl) was added to the maxi column to elute the DNA, allowing the solution to drain by gravity flow. The elution tube contains the purified DNA. 10.5ml isopropanol was added to the DNA and mixed well. The elution tube was centrifuged at >12,000 x g for 30 minutes at 4°C, carefully removing and discarding the supernatant. 5 ml 70% ethanol was then added to resuspend the DNA pellet and the tube was centrifuged at >12,000 x g for 5 minutes at 4°C, carefully removing and discarding the supernatant. After air-drying the pellet for ~10minutes, the DNA pellet was resuspended in 500µl TE Buffer (TE, 10 mM Tris-HCl, pH 8.0 0.1 mM EDTA) and the purified DNA was stored at -20°C.

2.2.5. Enzymatic Digestion

DNA eluted from Maxi and Mini Plasmid preparation was enzymatically digested and separated by agarose gel electrophoresis to determine the quality and the correct identification of the purified DNA. As the DNA sequence is known, the number and location of specific sites at which enzymes “cut” the DNA is also known, allowing the size of the DNA fragments measured by gel electrophoresis to be compared to the expected size of DNA fragments determined by calculation. If the size of the fragments seen on the gel differed from those calculated, a mutation had likely occurred during DNA replication and the DNA could not be used. For Mini Plasmid Preparations in which the DNA concentration is low, 4 µl of DNA was enzymatically digested. For Maxi Plasmid Preparations in which the DNA concentration is higher, 1µl of DNA from Maxi Plasmid Preparation was enzymatically digested. Additionally 0.5 µL (NotI, NheI, HindIII, ClaI) and Buffer H (10x stock, Cla I, Not I, Xho I) and / or Buffer B (10x stock, Bam H I, Xho I, Cla I) was added and made up to 10 µl with MilliQ water then incubated for 1hour at 37 °C. To prepare the
agarose gel, 1 g (1%) agarose (Invitrogen 16500100) was dissolved in 100 ml 0.5 x TBS by heating in the microwave for 1 minute, then allowing it to cool. The agar was poured into an electrophoresis small tray and comb was placed in gel, following setting ~20 minutes. 1-2 µl loading dye was added to each digest and 15 µl digest was added to each well in gel, as well as 5 µl of DNA ladder. The gel was run for one hour in TBE (89 mM Tris, 89 mM Boric acid, 2 mM EDTA, pH:8.0) at 90-100 volts. The Bands in each gel were compared to the expected size of DNA to confirm the DNA preparation was correct.

![Image of agarose gel](image)

**Figure 2-1 Example gel of βSAP97.**

1. DNA ladder, 2. uncut DNA. 3. βSAP97 cut with Hind III. 4. uncut βSAP97 DNA. 5. βSAP97 cut with Hind III (10x dilution). For lanes 3 and 5 seven bands are observed on sizes ~5000kb, ~2500kb, ~2000kb ~1200kb, ~1000kb, ~600kb and ~500kb which are similar to the expected band sizes of 5030 kb, 2466 kb, 1772kb, 1196kb, 1040kb, 584kb and 556kb.

### 2.3. Calcium Phosphate Transfection

Primary hippocampal culture neurons were transfected by calcium phosphate precipitation at DIV 7-8 and used for experiments at DIV 9-14. In a Class II hood, for each
35 mm culture well, 60 µl 2x HBS (10 mM KCl, 274 mM NaCl, 1.4 mM Na₂HPO₄, 15 mM glucose, 42 mM HEPES, pH 7.1) was added to a 15 ml polystyrene conical Falcon tube (Falcon 2095). 7.5 µl 2M CaCl₂, 2 µg DNA and sterile milliQ water (total volume 60 µl) was added to another tube. Using a pipetman, this DNA mix was added drop wise to the 2xHBS and tapped several times on the tube after each drop to mix well. Coverslips were transferred into 1 ml of pre-warmed 37°C Neural Basal Media with 10 µM CNQX and 50 µM APV added for neuroprotection. After 20 minutes of incubation in the dark at room temperature, the precipitate was pipetted onto the cultured neurons in 1 ml conditioned medium containing 10 µM CNQX and 50 µM APV. Cultured neurons were incubated for 25 minutes at 37°C and 5% CO₂ to allow DNA uptake. Transfection was stopped by rinsing the neurons three times with prewarmed HBSS (H2387, Sigma, pH 7.2) and the coverslips were then returned to their original culture media.

2.4. Immunocytochemistry

2.4.1. Fixation

Hippocampal neurons were washed twice in sterile 1x PBS at room temperature and fixed in 4% formaldehyde with or without 4% sucrose for 20 minutes. Neurons were washed once more in 1xPBS for 5 minutes to remove fixative. Neurons were either immuno stained immediately or stored for no more than 4 weeks at 4°C in 1xPBS + one drop 4% formaldehyde to maintain sterility.

2.4.2. Primary Antibody immunostaining

Fixed neurons were washed twice for 5 minutes at room temperature in non-sterile 1xPBS. Fixed neurons were permeabilised with 0.25% Triton X-100 for 5 minutes at room temperature to allow antibody penetration to bind their intracellular antigens. Neurons were washed twice for 5 minutes at room temperature in non-sterile 1xPBS and incubated at 37°C for 30 minutes in prewarmed blocking solution (2% bovine serum albumin, 2% gelatine, 2% glycine, 50 mM NH₄Cl). Coverslips were inverted onto primary antibody solution (antibody + blocking solution) for two hours at room temperature or at 4°C overnight.

2.4.3. Secondary Antibody immunostaining

Coverslips were rinsed once in non-sterile 1xPBS and washed three times for 10 minutes in non-sterile 1xPBS. In the dark to prevent photobleaching, coverslips were inverted onto fluorescently tagged secondary antibody diluted in 1xPBS for one hour at room temperature. Coverslips were rinsed once in non-sterile 1xPBS and were washed 3 x
10 minutes in non-sterile 1x PBS to remove any unbound antibody. Coverslips were mounted onto glass slides (City Fluor or Vectashield H-1000) and sealed with nail polish around the coverslip edges. Coverslips were stored at 4°C in the dark.

2.5. Image Processing

Image processing and analysis were performed using Image J (Rasband, 1997-2005). Images were acquired under oil immersion using a Zeiss Axioscope with a cooled charge couple device (CCD) digital camera in the Biomedical Imaging Research Unit (BIRU), Faculty of Medical and Health Sciences, University of Auckland. The filters used for each of the secondary antibodies were DAPI (bandpass barrier filter) for Alexa 350 and AMCA, GFP (bandpass barrier filter) for Alexa 488, Rhodamine for Alexa 568 and Cy5 for Alexa 647. For each neuron between one and four 12-bit (0-4095) z-stacks were acquired of dendrites at 63x magnification (63) as well as a z-stack of the whole neuron at 40 x magnification. Only 63x magnification images were used for dendritic analysis. Distances between planes in the z-stack were 0.5 µm at 63 x magnification, 1.0 µm for 40 x magnification and 2.0 µm for 20 x magnification (without oil). A binning of two was used to reduce exposure times and therefore photo-bleaching. Exposure times were chosen based on the specimen intensity so that the full dynamic range was acquired. However, all imaging parameters including exposure times within each experiment were kept constant to ensure intensity measures would be directly comparable. Z-stacks were separated into their different channels and were changed from 12 bit to 8 bit images (0-255). The z-stacks were merged into maximum projection images and out of focus z stacks were removed. When z-stacks were merged into maximum projection images, two puncta or spines could merge into one if they lay in the same x-y coordinate but at different heights in the z-stack. Therefore dendrite analysis could be improved by 3D reconstruction, but dendrites are thin structures and so merging of two objects into one would not happen significantly to influence the data. Background subtraction was conducted using a rolling ball radius of 50. All spines and puncta on dendrites were included in the dendritic regions for analysis and puncta density was determined by manually counting the density along the length of spines. Intensity values were acquired by a puncta-by-puncta analysis.

2.6. Whole cell patch clamping

Whole-cell patch-clamp recordings were conducted on neurons in hippocampal dissociated culture. Cultured neurons were transferred to a recording chamber (volume
approximate 1 ml) superfused at 2–3 ml/min with artificial cerebrospinal fluid (ACSF; in mM: 120 NaCl, 1.25 NaH₂PO₄, 3 KCl, 2 CaCl₂, 2 MgSO₄, 20 D- (+)-glucose, 26 NaHCO₃) bubbled with Carbogen (95%O₂/5% CO₂) at room temperature (21–23°C). Neurons were visualised using a Zeiss Axioskop 2FS microscope on a vibration isolation table with a CCD/R video camera (SonyxC-E 150) and a Hamamatsu camera controller (C2741-60) to adjust contrast on the video monitor (Xpose L-7 DSV). Whole-cell patch-clamp was conducted using differential interference microscopy (DIC). All equipment were electrically grounded through a transformer (Tridonic. Atco TX 2000FPE-01). Glass electrodes with resistances between 4-10MΩ were pulled using a horizontal electrode puller (P-97, Sutter Instruments, USA). Glass electrodes were fabricated from KG-33 capillary glass without filament (OD=1.65, ID=1.15 Length 4 inches: Garner Glass DA-S300230). Electrode position was adjusted and controlled using a micromanipulator (Sutter MPC-200). The internal pipette solution consisted of (in mM): K gluconate (presynaptic) or Cs gluconate (postsynaptic), 120; HEPES, 40; MgCl₂, 5; NaATP, 2; NaGTP, 0.3; pH 7.2 with KOH or CsOH. Pipette solution and ACSF solution osmolarity were routinely measured (280-290mOsm and 300mOsm respectively). All whole-cell patch-clamp experiments were conducted at room temperature and membrane currents and potentials processed with a Multiclamp 700B commander (Molecular Devices) and digitized at 10KHz using a Digidata 1322 (Molecular Devices) to convert analogue to digital signals. Events were sampled at 10KHz and low-pass filtered at 1 KHz. Series resistance was measured (Rs) and recordings with Rs variation above 20% were discarded from the data analysis. Data acquisition and analysis was performed using AxoGraph X (AxoGraph Scientific) and pClamp 9 (Molecular Devices) software. In dual whole cell patch clamp recordings, presynaptic neurons (control untransfected cells) were held in current clamp and postsynaptic cells (α- or βSAP97–EGFP transfected neurons) were held in voltage clamp at -65mV.

Presynaptic neurons were induced to fire single action potentials by a 20 ms current pulse (typically 20-50 pA). Synaptically connected pairs were identified by the presence of monosynaptic AMPA receptor-mediated excitatory postsynaptic currents (EPSCs) induced within 3 ms of the peak of the presynaptic action potential (Pavlidis & Madison, 1999; Montgomery et al., 2001). Isolated NMDAR EPSCs were measured at +40 mV in ACSF containing 10 µM NBQX. Polysynaptic inhibitory currents were identified by their longer latency and reversal potential at ~50mV and were excluded from data analysis. Baseline AMPAR-and NMDAR-mediated EPSCs in response to presynaptic action potential firing were collected at 0.1 Hz. Sweeps in which no presynaptic action potential occurred or in which the postsynaptic recording was distorted by spontaneous synaptic activity were
excluded from analysis. In some experiments, polysynaptic events obscured the peak of the event.

For synaptic plasticity induction, NMDA / glycine (chemical LTD: 25µM NMDA + 10µM glycine + 1µM strychnine) or bicuculline / glycine (chemical LTP: 20µM bicucullin + 200µM glycine + 1µM strychnine) was used to induce cLTD or cLTP. 5 minutes of baseline AMPAR-mediated EPSCs were first collected followed by 5 minute application of cLTP or cLTD solution. Paired whole cell recordings were held for a minimum of 30 minutes.

In some experiments, long-term depression was also induced by low-frequency presynaptic action potentials at 1 Hz paired with postsynaptic cell depolarization to ~55 mV for the time indicated. Long-term potentiation was induced by pairing presynaptic action potential a 1 Hz with postsynaptic depolarization to -10 to 0 mV. Spontaneous miniature EPSCs (mEPSCs), were recorded in aCSF containing 1µM TTX and 110µM picrotoxin to eliminate evoked excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs), respectively. For mEPSCs, data were acquired continuously until a minimum of 100 mEPSCs were recorded. mEPSC events were detected with MiniAnalysis (Synaptosoft; version 6.0.3).

To measure surface AMPA and NMDA receptor-mediated currents (both synaptic and non-synaptic), exogenous AMPA (1mM) and NMDA (1mM) were applied from a micropipette 200µm from the dendrites of α- or βSAP97–EGFP transfected neurons with a picospritzer (pressure 2 bar; pulse 200 ms). To verify that the exogenous AMPA and NMDA was not saturating the receptors, therefore preventing any increase in AMPA and NMDA currents being measured, an additional application of AMPA and NMDA was applied closer to the dendrites at the completion of each experiment to verify the current amplitude could be further increased. All solution protocols are in Table 2.5-1 to Table 2.5-5.

2.6.1. Analysis

Data analysis of evoked EPSCs was performed using AxoGraph X. To detect potentiation of postsynaptic currents and synaptic input a sliding template function was used. For postsynaptic currents a template was used with the following parameters: Baseline 1ms, length 20ms. Template 1: 40,000µV, 2ms rise time, 4 ms decay time; Template 2: 50,000 µV, 2 ms rise time, 4 ms decay time. Events were detected with a threshold of 1, captured 5 ms baseline and 20 ms length 0 and baseline for analysis of amplitude.

For synaptic inputs recorded in voltage clamp the miniature excitatory postsynaptic postsynaptic current (mEPSCs) function was used with 1 template with the following parameters: Baseline 10 ms, length 500 ms, 100 ms event separation; Template 1 : -100
pA, 50 ms rise time, 150 ms decay time. Events were detected with a threshold of -1, captured (10ms baseline and 500 ms length) and baselined for analysis of amplitude.

2.6.2. Statistics

Student t-tests were performed, two-tailed distribution, unequal variance, to determine whether results were statistically different. All data are presented with 2 decimal places plus or minus (±) the standard error of the mean (SEM) where n=number of neurons analysed. Statistical significance of changes in AMPAR and NMDAR EPSC amplitudes was tested with a level of significance set at $p < 0.05$. Since the mEPSC amplitude distribution did not follow a normal distribution, the nonparametric Kolmogorov–Smirnov test (Van der Kloot, 1991) was used to determine the probability of a significant difference between current amplitudes measured in control and SAP97-transfected neurons.
### Table 2-1 Artificial Cerebrospinal Fluid (ACSF)

<table>
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<tr>
<td>NaH$_2$PO$_4$</td>
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</tr>
<tr>
<td>MgSO$_4$</td>
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<tr>
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<tr>
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### Table 2-2 Potassium Gluconate Internal Solution

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<th>Concentration (mM)</th>
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</tr>
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<tr>
<td>MgCl$_2$</td>
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</tr>
<tr>
<td>NaGTP</td>
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### Table 2-3 Caesium (Cs)Gluconate Internal Solution

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<tbody>
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### Table 2-4 Chemical LTP (cLTP) Solution

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<tr>
<td>Bicuculline</td>
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</tr>
<tr>
<td>Chemical</td>
<td>Concentration (µM)</td>
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<td>---------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Glycine</td>
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</tr>
<tr>
<td>Strychnine</td>
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</tr>
<tr>
<td>NMDA</td>
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</table>
Chapter 3. SAP97 isoforms affect AMPA receptor-mediated synaptic transmission in hippocampal neurons

3.1. Introduction

Controlling AMPAR insertion and removal from the postsynaptic density (PSD) is a major mechanism underlying the expression of synaptic plasticity, providing a potential molecular basis for learning and memory. The GluR1 subunit of AMPARs is of particular interest due to its importance in the expression of LTP and LTD at synapses (Ehrlich et al., 2007; Citri & Malenka, 2008). SAP97 is a GluR1 binding partner but its role in the trafficking and synaptic localization of GluR1 during baseline synaptic transmission and synaptic plasticity is unclear. SAP97 has been proposed to drive GluR1 to synapses and overexpression of SAP97 increases the amplitude and frequency of AMPA receptor-mediated excitatory postsynaptic currents (EPSCs), while shRNA- knockdown of SAP97 reduces AMPAR surface expression and EPSC amplitude (Rumbaugh et al., 2003; Nakagawa et al., 2004). However, other investigators have reported that overexpression or loss of SAP97 had no effect on AMPAR-mediated neurotransmission (Schnell et al., 2002; Tomita et al., 2003; Ehrlich & Malinow, 2004; Schluter et al., 2006). Much of this controversy likely stems from the molecular diversity of SAP97, created by extensive alternative splicing and a general lack of knowledge about the subcellular distributions and functions of individual SAP97 isoforms. SAP97 is alternatively spliced between the N-terminal L27 domain and the first PDZ domain, and between its SH3 and guanylated kinase domains. The N-terminus splicing of SAP97 results in the inclusion of either an L27 domain (β-isoform) or containing a palmitoylation motif (α-isoform). This N-terminal splicing of SAP97 has functional consequences for the trafficking of SAP97 and for their roles in synaptic function. αSAP97 was shown to increase synaptic AMPARs and rescue the shRNA-mediated knockdown effect of PSD95 on AMPAR mediated transmission in slice cultures (Schluter et al., 2006). Interestingly, βSAP97 did not share these properties, indicating that splicing endows SAP97 isoforms with different characteristics (Muller et al., 1995; Lee et al., 2002; Schluter et al., 2006; Waites et al., 2009). However, when and where SAP97 isoforms interact with AMPA receptors and whether they direct the insertion or the retention of AMPARs within the PSD is elusive.

In the present study, we aimed to evaluate the functional properties of α- and β SAP97 isoforms by studying their effects on synaptic AMPARs. We hypothesized that (1)
Overexpression or shRNAi of α- or βSAP97 would alter the amplitude or frequency of AMPA-mediated mEPSCs; (2) Overexpression of α- and β SAP97 would alter the amplitude of evoked AMPAR-mediated EPSCs; (3) Knockdown of PSD95 and subsequent rescue with α- or βSAP97 would further reveal specific roles of α- and βSAP97 of AMPAR-mediated synaptic transmission in hippocampal neurons. In these experiments, we used whole cell patch clamping from single hippocampal neurons to record mEPSCs. In addition, we have used paired whole cell patch clamp recordings between presynaptic and postsynaptic cells in which postsynaptic cells were transfected with α- and βSAP97-EGFP, or ΔSAP97–EGFP lacking both the initial 10 aa residues of αSAP97 (including its putative palmitoylation motif) and the initial 106 residues (including the L27 domain) of βSAP97. Our data show that βSAP97 decreases the amplitude of synaptic AMPAR-mediated EPSCs and increases the surface AMPA-mediated currents. In contrast, αSAP97 increase the amplitude of synaptic AMPAR-mediated EPSCs and decreases the surface AMPA-mediated currents. These data show α- and βSAP97 have distinct effects on AMPAR-mediated EPSC. These data were published in the Journal of Neuroscience in April, 2009, entitled “Synaptic SAP97 Isoforms Regulate AMPA Receptor Dynamics and Access to Presynaptic Glutamate” (Waites et al., 2009). All the data in the results section of Chapter 3 are from experiments performed by me. Some of these data are published in Waites et al., (2009). The data in the discussion were acquired at Stanford University.

3.2. Results

3.2.1. SAP97 isoforms significantly alter miniature and evoked AMPAR EPSCs

Overexpression of α- and βSAP97 shows distinct subcellular localization patterns in hippocampal neurons. Both isoforms have punctate dendritic distributions (Fig. 3.1). However, whereas α SAP97–EGFP was highly enriched in dendritic spines, the β- isoform was found both in spines and more diffusely throughout dendritic shafts and cell bodies. Because of their potential yet controversial roles in regulating AMPAR-mediated EPSCs (Sans et al., 2001; Rumbaugh et al., 2003; Nakagawa et al., 2004; Schluter et al., 2006; Waites et al., 2009), we were specifically interested in evaluating the localization and dynamics of the N-terminal isoforms α- and βSAP97 containing the same set of additional inserts (I1b/I3/I5) (Chapter 1, Fig. 1.3).

We first examined whether α- and β SAP97 influence AMPAR-mediated spontaneous miniature currents in hippocampal neurons. We chose ΔSAP97–EGFP as a special ‘control’, because the more restricted synaptic localization and slower exchange rate observed (Waites et al., 2009) for αSAP97 compared with βSAP97 could be attributable to the presence of its putative palmitoylation domain and/or to the lack of the
Figure 3-1 Subcellular localization of α- and βSAP97 isoforms.

In mature hippocampal cultures, overexpression of α- and βSAP97 by calcium phosphate precipitation at DIV 7–9 revealed both isoforms had punctate dendritic distributions. A: αSAP97–EGFP was highly enriched in dendritic spines; B: the β-isoform was found both in spines and more diffusely throughout dendritic shafts and cell bodies. Scale bars: 20µm.
L27 domain. Therefore, it is a possibility that the N-terminal domains could affect the frequency and amplitude of mEPSC. To distinguish between these possibilities, AMPAR-mediated mEPSCs were collected by whole cell patch clamp recordings from dissociated hippocampal neurons transfected with α-, βSAP97 EGFP, or soluble EGFP or ΔSAP97-EGFP. Miniature EPSC frequency and amplitude was not significantly different between ΔSAP97-EGFP and control EGFP expressing neurons (Fig. 3.2. A-C; ΔSAP97 amplitude: 16.0 ± 0.34 pA, frequency: 1.23 ± 0.54 Hz, n=8; control amplitude: 15.3 ± 0.27 pA, frequency: 1.20 ± 0.25 Hz, n= 6). In contrast, αSAP97 and βSAP97 differentially altered mEPSC amplitude and frequency. Overexpression of αSAP97-EGFP significantly increased mEPSC amplitude compared with control (Fig.3.2 A-C. αSAP97 amplitude: 18.4 ± 0.7 pA; n= 5; control amplitude: 15. 3 ± 0.27 pA; n=6; p<0.05; control frequency: 1.20 ± 0.17 Hz; n=6; αSAP97 frequency: 1.63 ± 0.38 Hz; n=5; p<0.05). However, βSAP97-EGFP significantly decreased mEPSC amplitude compared with control levels (Fig.3.2. A-C. βSAP97 and control amplitude respectively:13.7 ± 0.17 pA; n=7; 15.1 ± 0.21 pA, p<0.05; control and βSAP97 frequency respectively :1.22 ± 0.23 Hz: 0.29 ± 0.09 Hz; p< 0.05). These results showed that αSAP97 and βSAP97 have opposite effects on AMPAR-mediated mEPSCs in hippocampal neurons.

Further investigation of the effects of αSAP97 and βSAP97 on synaptic transmission was conducted by assessing synaptically evoked AMPAR-mediated EPSCs (Fig. 3.2. D,E). Paired whole-cell recordings were performed between hippocampal primary neurons in dissociated cultures with the postsynaptic neuron expressing soluble EGFP, αSAP97 or βSAP97. The amplitudes of evoked EPSCs were significantly increased in hippocampal neurons overexpressing αSAP97-EGFP compared to control untransfected neurons (Fig. 3.2. D,E. 472 ±128 pA, n=13 paired recordings; control: 256 ± 40 pA, n=23 paired recordings, p < 0.05). However, the amplitude of AMPAR-mediated evoked EPSCs was significantly decreased in hippocampal neurons overexpressing βSAP97-EGFP neurons relative to control cells (Fig. 3.2. D,E. 87 ± 17 pA, n<10; p <0.01). Together, the mEPSC and evoked EPSC data indicate that αSAP97 is able to recruit functional AMPARs to the PSD as showed previously (Rumbaugh et al., 2003; Schluter et al., 2006). Whereas βSAP97 decreased the number of AMPARs in the PSD and it was not able to recruit the number of AMPARs to synapses.
Figure 3-2 SAP97 isoform-specific effects on miniature and evoked AMPAR currents.

A, Sample miniature EPSC traces recorded from primary hippocampal neurons transfected with soluble EGFP (control) or α-,β- or Δ-SAP97–EGFP. B, Cumulative probability plot of mEPSC amplitudes from these neurons. The Kolmogorov–Smirnov test determined a significant effect of α- and βSAP97–EGFP (p<0.001) but not ΔSAP97–EGFP on mEPSC amplitudes. Arrowheads indicate the maximum mEPSC amplitudes. C, Histogram depicting the same mEPSC amplitude data (400 mEPSC events per construct). Note the right shift of the histogram for neurons expressing αSAP97–EGFP and the left shift for those expressing βSAP97–EGFP. D, Evoked EPSC sample traces from postsynaptic neurons transfected with α- and βSAP97–EGFP and untransfected controls (10 superimposed sweeps per condition). E, Quantification of AMPAR EPSC amplitudes (means±SEM; n=10 paired recordings).
Previous studies showed that knockdown of PSD-95 by RNAi significantly reduces AMPAR-mediated EPSCs and spine density (Nakagawa et al., 2004; Elias et al., 2006; Schluter et al., 2006; Ehrlich et al., 2007; Chen et al., 2011). In order to determine the ability of SAP97 isoforms to rescue the phenotype produced by knockdown of PSD-95, and consequently provide information on how SAP97 isoforms target AMPA receptors in the synaptic membrane, we examined the effects of α- and βSAP97 on AMPAR-mediated mEPSCs caused in hippocampal neurons in which PSD-95 has been knocked down. AMPAR-mediated miniature EPSCs were recorded from hippocampal neurons which were transfected with constructs expressing sh95 with soluble EGFP, or sh95 together with EGFP-tagged α- or βSAP97. As reported previously (Rumbaugh et al., 2003; Schluter et al., 2006), expression of sh95 alone caused a near-complete loss of AMPAR-mediated currents, reflected by a dramatic reduction in mEPSC frequency (Fig. 3.3. A, B. Control frequency: 1.54 ± 0.37 Hz, n=32; sh95 frequency: 0.13± 0.11 Hz, p < 0.001, n=14).This phenotype was rescued by αSAP97 expression, which restored mEPSC frequency to control levels (Fig. 3.3. A, B. 1.15± 0.42 Hz; n=12) but not by βSAP97 expression (Fig. 3.3. A,B. 0.06 ± 0.03 Hz; n= 18; p < 0.001). One explanation for this result is that βSAP97 does not localize AMPA receptors to the PSD and instead holds AMPARs in an extrasynaptic domain of the plasma membrane. An alternative explanation is that βSAP97 decreases the channel conductance of AMPARs and decreases the functional AMPARs in the PSD thus dramatically decreasing mEPSCs.

To distinguish these possibilities, we examined the amplitude of AMPAR-mediated currents in hippocampal neurons overexpressing α- or βSAP97–EGFP by focal application of exogenous AMPA. This exogenous AMPA application will activate both synaptic and extrasynaptic AMPARs. If there is the presence of higher amplitude AMPAR-mediated currents in βSAP97 expressing neurons, it would support that βSAP97 held AMPARs in the extrasynaptic domain of plasma membrane. The absence of such currents would suggest impaired channel conductance underlie the βSAP97 phenotype. We found that the peak amplitude of AMPA induced currents in βSAP97-expressing neurons was significantly higher than that seen in αSAP97-expressing neurons (Fig. 3.3.C. βSAP97: 232.3 ± 32.6 pA, n=9; αSAP97: 116.31 ± 12.8 pA, n= 9, p < 0.005). Taken together, the above electrophysiological data showed that overexpression of βSAP97 induces greater total numbers of surface AMPARs, but that these receptors are not localised at the synaptic sites.
Figure 3-3 Isoform-specific rescue of PSD-95 knockdown phenotype.

A, Sample mEPSC traces from untransfected control neurons (top) and those coexpressing sh95 together with soluble EGFP (sh95), αSAP97-EGFP or EGFP βSAP97. B, Quantification of mEPSC frequency data from these cells. The Mann–Whitney Rank sum test determined a significant effect (p<0.001) of sh95 and EGFP- βSAP97 plus sh95 but not αSAP97–EGFP plus sh95 on mini-EPSC frequency. C, Sample traces of inward AMPAR currents induced by focal AMPA application in α- (top) or βSAP97–EGFP (bottom)-transfected neurons.
We also examined AMPAR-mediated miniature EPSCs in hippocampal neurons expressing shRNAiSAP97 in which the expression was downregulated with a short hairpin RNA of all SAP97 isoforms (sh97). We found shSAP97 did not significantly affect AMPA-mediated mEPSC frequency and amplitude (Fig. 3.4. sh97 and control frequency separately: $2.3 \pm 0.2$ Hz, n=5; $2.5 \pm 0.3$ Hz, n=6, p>0.05; sh97 and control amplitude separately: $12.8 \pm 0.5$ pA, n=5; $13.4 \pm 0.3$ pA, n=6; p>0.05). This data is consistent with other reports (Schnell et al., 2002; Tomita et al., 2003; Ehrlich & Malinow, 2004; Schluter et al., 2006). This finding raises the possibility that DLG-MAGUK family members have functional redundancy and compensate for each other so that the loss of SAP97 will be compensated by other family members. Alternatively, other adaptive processes during synaptogenesis and synapse maturation might compensate for the loss of the normal function of DLG-MAGUKs in basal synaptic transmission (Nakagawa et al., 2004; Schluter et al., 2006).
Figure 3-4  Comparison of average mEPSC frequency and amplitudes from sh97 and control.

A: Sh97 did not change the frequency of mEPSCs in sh97 expressing neurons, indicating that overexpression of sh97 did not affect presynaptic glutamate release probability; B: Sh97 did not change mEPSC amplitude in sh97 expressing neurons, indicating that overexpression of sh97 did not affect synaptic AMPARs. ‘ns’ means no significance.
3.3. Discussion

3.3.1. The role of αSAP97 in regulating the functional AMPARs in dendritic spines

In this study, the ability of α- and βSAP97 isoforms to directly regulate the functions of AMPARs within spines was examined. Here we have shown that α or βSAP97 overexpression significantly alters miniature and evoked AMPAR-mediated current amplitude and frequency in opposite directions. Focal application of exogenous AMPA revealed that peak amplitudes of AMPA-induced currents were significantly smaller in neurons expressing αSAP97 compared with βSAP97. In combination with the αSAP97 induced increase in AMPAR EPSC amplitude and the βSAP97 induced decrease in AMPAR EPSCs, these data suggest that αSAP97 shifts extra-synaptic AMPARs to the synapse, while βSAP97 recruits AMPARs to extrasynaptic sites.

3.3.1.1. Sh95 reveals opposite effects of α- and β SAP97 on AMPARs

To determine whether the N-terminal domain of SAP97 governs its effects on AMPAR EPSCs, we examined the effect of overexpression of αSAP97 and βSAP97 with sh95 in hippocampal neurons. This enabled us to determine the potential role of αSAP97 independent of PSD95. The data showed that αSAP97 rescued and restored the loss of AMPAR-mediated EPSCs caused by PSD-95 knockdown in dissociated hippocampal neurons. The “rescue” of the sh95-mediated synaptic depression by αSAP97 suggests that this isoform alone is capable of mediating the increase in AMPAR-mediated synaptic strength, as the sh95 silences both α- and β-isoforms of PSD-95. This behaviour was also observed in organotypic slices, in which αSAP97 dramatically increased AMPAR EPSCs 2- to 3-fold when either overexpressed or replacing endogenous PSD-95 (Schluter et al., 2006).

We also examined whether βSAP97 can rescue the loss of AMPA mEPSCs in neurons lacking PSD95 and found that βSAP97 did not rescue the loss of AMPAR mEPSCs seen in neurons lacking PSD-95. Due to functional redundancy and compensation in the MAGUK family, acute βSAP97 overexpression should compensate for the loss of PSD95 if βSAP97 is able to recruit new functional AMPARs into the PSD. We speculated that βSAP97 may localize to extrasynaptic sites because it lacks a palmitoylation domain and cannot compete with PSD-95 for binding sites within the PSD. However, in the absence of PSD-95, binding sites may become available within the PSD, enabling βSAP97 to enter this compartment, recruit GluR1-containing AMPARs, and thus
rescue the PSD-95 knockdown phenotype. However, this did not occur as βSAP97 could not rescue AMPAR-mediated synaptic transmission in sh95 neurons.

The near-complete loss of AMPAR-mediated currents in sh95 expressing neurons reflects PSD95 having a central role in stabilising AMPARs in the PSD. Indeed, PSD-95 expression enhances postsynaptic clustering and activity of glutamate receptors (El-Husseini et al., 2000), and overexpression of PSD-95 in hippocampal neurons drives maturation of glutamatergic synapses (El-Husseini et al., 2000; Rumbaugh et al., 2003; Nakagawa et al., 2004). Postsynaptic expression of PSD-95 also enhances maturation of the presynaptic terminal through conveying a retrograde signal for presynaptic development and PSD-95 expression. Together these data show that PSD-95 has a central role in synaptic development, synapse stabilization and plasticity (El-Husseini et al., 2000).

Our findings point to a significant functional redundancy between αSAP97 and PSD-95. One notable difference is that the former interacts directly with GluR1. GluR1 binds to the second PDZ domain of SAP97 but not to the PDZ domains of PSD-93, PSD-95, or SAP102 (Cai et al., 2002). In contrast, PSD95 interacts with GluR1 via a transmembrane AMPAR regulatory protein (TARP)-dependent mechanism (deSouza & Ziff, 2002; Tomita et al., 2003; Nicoll et al., 2006). Knockdown of PSD95 will dissociate the interaction with AMPARs and prevent TARPs recruiting GluR1 to the PSD. Overexpression of the TARP stargazin in hippocampal slice cultures selectively augments the number of extrasynaptic AMPA receptors and clustering of these additional receptors to the synapse requires increased levels of the synaptic stargazin anchor, PSD-95 (Schnell et al., 2002; Tomita et al., 2003). Interestingly, the PDZ-binding site of stargazin contains a consensus site for numerous neuronal protein kinases, and phosphorylation of this site disrupts interaction with PSD-95 and prevents synaptic clustering of AMPA receptors (Choi et al., 2002; Tomita et al., 2003). Additional experiments are needed to investigate the functional relevance of these interactions, which could relate to a more fundamental role of PSD-95 in maintaining synaptic pools of GluR2/3, although this has not been clearly established (Ehrlich & Malinow, 2004; Boehm et al., 2006a). According to our data and other studies e.g. Schluter et al., (2006), we propose αSAP97 is an important “backup” of αPSD95. When αPSD95 is down regulated physiologically or pathologically, αSAP97 could replace αPSD95 and stabilize synaptic transmission. Consistent with our hypothesis, LTP is strongly expressed in PSD-95 knockout animals (Migaud et al., 1998).

3.3.2. Role of βSAP97 in the localization of AMPARs

We examined the effect of βSAP97 in AMPAR-mediated synaptic transmission. Unexpectedly, we found that βSAP97–EGFP significantly decreased the mEPSC amplitude
and frequency. This data is the opposite to some previous reports (Nakagawa et al., 2004; Schluter et al., 2006; Howard et al., 2010). Our data suggest that βSAP97 decreases the number of functional AMPARs within the PSD, and/or that overexpression of βSAP97 changed presynaptic function resulting in decreased glutamate release at the presynaptic terminal. According to previous studies (Regalado et al., 2006), overexpression of βSAP97 enhances the size of presynaptic boutons through transsynaptic signalling. Therefore it is possible our observed electrophysiological characteristics are due to changing the presynaptic size or function by transsynaptic signalling in α- and βSAP97 overexpressing neurons. However, parallel experiments to this study were conducted by our Stanford colleagues to test synaptic vesicle recycling by FM4-64 uptake and have ruled out the change of presynaptic function. Their data showed α-and βSAP97 have identical effects on juxtaposed presynaptic boutons, with both α-and βSAP97–EGFP significantly increasing the size of the total recycling pool of synaptic vesicles as assessed by FM4-64 uptake. Furthermore, neither isoform altered the synaptic vesicle exocytosis kinetics at juxtaposed boutons, as measured by FM4-64 destaining rates. These data indicate that transsynaptic effects cannot account for the dramatic differences in AMPAR EPSC amplitudes seen in neurons expressing α- and βSAP97. Therefore, our data suggest that βSAP97 decreases synaptic transmission through reducing the number of functional AMPARs within the PSD.

Our observed effects of βSAP97 on synaptically evoked AMPAR-mediated EPSCs confirm this conclusion. Once more, consistent with our mEPSC data, the amplitudes of evoked EPSCs were significantly decreased in pyramidal neurons expressing βSAP97-EGFP relative to neurons expressing αSAP97 and untransfected controls. Furthermore, our simultaneous recordings from two individual synaptically connected neurons enabled the direct analysis of synaptic transmission in very small populations of synapses. This technique endows examination of synaptic function at a closer level and elucidates more clearly the mechanisms of changes of neurons expressing SAP97. Using this technique avoids recording from large populations of synapses, which reflects an average synaptic response and cannot reveal whether all synapses may respond to the same stimulus in the same fashion. However, other investigators (Nakagawa et al., 2004; Schluter et al., 2006; Howard et al., 2010) used single cell patch clamp and stimulated extracellularly in stratum radiatum. The stimulation of stratum radiatum will stimulate a large population of synapses. Therefore the transfected cell and untransfected cell that they recorded from could separately receive different inputs from a different number of synapses. Thus, dual whole cell paired recordings minimises recording from heterogenous synapses and therefore enabled us to see the effect of βSAP97.

Furthermore, our findings showed that βSAP97 did not rescue AMPAR mediated currents in the absence of PSD-95. This was at odds with a previous study performed in
hippocampal organotypic slices (Schluter et al., 2006). A likely explanation for these differences is that the cell-surface levels of GluR1-containing AMPARs vary widely between hippocampal preparations. In dissociated cultures, GluR1 is readily detectable on the surface of cell bodies and dendrites, at synaptic and extrasynaptic sites, under both basal and stimulated conditions (Bats et al., 2007; Ehrlich et al., 2007; Waites et al., 2009). In organotypic slices, in contrast, the vast majority of GluR1 is localized intracellularly within dendritic shafts, only moving into spines during synaptic stimulation (Shi et al., 1999). Based on previous studies showing a direct interaction between βSAP97 and GluR1 but not GluR2/3 (Leonard et al., 1998; Cai et al., 2002; von Ossowski et al., 2006), one would predict that βSAP97 affects synaptic AMPAR currents under basal conditions in dissociated cultures but not in organotypic slices. Similarly, the ability of SAP97 to rescue the PSD-95 knockdown may depend on the subunit composition of cell-surface AMPARs in these two preparations. If the majority of surface receptors are GluR2/3, as in organotypic slices, βSAP97 may rescue the PSD-95 knockdown phenotype via an indirect, compensatory mechanism during AMPAR trafficking, or by facilitating the synaptic retention of GluR2/3 through TARPs (deSouza & Ziff, 2002; Ehrlich et al., 2007; Shepherd & Huganir, 2007), although this functionality of βSAP97 has not been explored. However, if the majority of cell-surface receptors contain GluR1, as in dissociated cultures, βSAP97 will sequester these receptors in an extrasynaptic domain and thus fail to rescue the PSD-95 knockdown phenotype as we observed.

In our experiments, the βSAP97 induced a decrease in the mEPSC amplitude and is possibly because βSAP97 interferes with AMPAR channel conductance. In other tissue, e.g. cardiac myocytes, SAP97 binds to the inwardly rectifying potassium channel, Kir2.3, and increases unitary conductance and cell surface expression of the channel without altering the channel open probability and current rectification properties (Vikstrom et al., 2009). This result implies that SAP97 can change channel conformation and further regulate channel currents. We observed that focal application of exogenous AMPA revealed that peak amplitudes of AMPA-induced currents were significantly greater in neurons expressing βSAP97 compared with αSAP97. This result indicates that channel conductance has not been blocked in neurons expressing βSAP97. However, further experiments examining AMPA application to AMPARs in α/β expressing HEK cells, is required to fully determine whether α- or βSAP97 more finely regulate AMPAR conductance.

In addition to using overexpression of βSAP97 in our experiments, we have examined the role of sh97 in hippocampal neurons. mEPSCs were recorded in sh97 expressing neurons and the data shows no significant changes in mEPSC amplitude or frequency. These data imply that there are mechanisms of functional redundancy and
compensation in MAGUK family, and the loss of SAP97 could be compensated by other family members (Schluter et al., 2006). Our sh97 data also suggest that SAP97 contributes little to basal synaptic transmission. This is consistent with a previous report which showed that endogenous PSD-95 has a dominant effect over SAP97 (Schluter et al., 2006). The function of SAP97 may therefore only best be revealed on a background of greatly reduced PSD-95. However, αPSD95 is the dominant PSD95 isoform in neurons and βSAP97 is the dominant SAP97 isoform (Schluter et al., 2006). Therefore, if βSAP97 indeed regulates extrasynaptic receptors, this would not be visible in measurements of synaptic mEPSCs in sh97 neurons. Further experiments examining extrasynaptic AMPARs in sh97 neurons are therefore required.

3.3.3. Properties of α- and βSAP97 as determined by parallel imaging experiments

In parallel to our electrophysiology experiments, our collaborators at Stanford University performed imaging experiments of α- and βSAP97 in hippocampal neurons. These data are summarised below and then discussed in relation to the electrophysiology data in section 3.3.4.

(1) To examine whether α- and βSAP97 regulate the surface expression of GluR1-containing AMPARs, the cell-surface levels of endogenous GluR1 in transfected neurons expressing α- or βSAP97–EGFP was determined. Endogenous GluR1 puncta that colocalized with α- and βSAP97–EGFP in dendritic spines were identified. Overexpression of α- and βSAP97 was found to increase the surface expression of endogenous and recombinant GluR1 within dendritic spines (Waites et al., 2009).

(2) To evaluate whether the differences in subcellular distribution between β- and αSAP97–EGFP reflected different dynamic properties, they examined SAP97 isoform dynamic behaviors within spines using fluorescence recovery after photobleaching (FRAP). The recovery of bleached β- or αSAP97–EGFP puncta was monitored for a 30 min period. Strikingly, the two isoforms exhibited dramatically different recovery rates, with less than 50% of αSAP97–EGFP fluorescence and more than 80% of βSAP97–EGFP fluorescence recovering within this time window (Waites et al., 2009). These data indicate that αSAP97 is more stably associated with postsynaptic structures than βSAP97.

(3) By comparing the SAP97–EGFP fluorescence intensity of individual synaptic puncta before and after Triton X-100 extraction, they were also able to estimate the insoluble fraction of each SAP97 isoform. Many PSD and cytoskeletal associated proteins are insensitive to Triton extraction, whereas cytoplasmic and membrane-associated proteins are solubilized and washed out in dissociated hippocampal culture. For example,
spine PSD-95 is resistant to extraction buffer containing Triton X-100, indicating PSD95 is stable in the PSD (Sharma et al., 2006). As expected from their FRAP experiments, the majority (~60%) of αSAP97–EGFP was Triton resistant, suggesting an association with lipids and proteins within the PSD. In contrast, only ~30% of one-third of βSAP97 is associated with the cortical cytoskeleton and/or the PSD, whereas a majority of ΔSAP97, lacking both the initial 10 aa residues of αSAP97 including its putative palmitoylation motif and the initial 106 residues including the L27 domain of βSAP97, is associated with neither (Waites et al., 2009).

(4) To more closely examine the subsynaptic distributions of α- and βSAP97, they performed silver-enhanced immunogold EM of neurons expressing α- or βSAP97–EGFP. Quantitative analysis of EM micrographs revealed that within spines, ~50% of silver-enhanced gold particles labeling αSAP97–EGFP were associated with the PSD, whereas only ~30% of gold particles labeling βSAP97–EGFP could be detected at the PSD. In contrast, the majority (~70%) of gold particles labeling βSAP97–EGFP appeared at other locations, most commonly near the spine plasma membrane or within the cytoplasm, that were often adjacent to the PSD. These data are consistent with other immuno-EM studies showing that L27 domain containing SAP97 isoforms have a predominantly peripheral PSD distribution (DeGiorgis et al., 2006; Aoki et al., 2001).

3.3.4. α- and βSAP97 create docking sites for AMPARs within postsynaptic compartments

Together the live cell imaging and immunocytochemistry experiments show that α- and βSAP97 promote the postsynaptic surface accumulation of GluR1 but in different compartments. Importantly, both isoforms slow GluR1 exchange by increasing the fraction of tightly bound mCh–GluR1 rather than altering the rate constants of mCh–GluR1 exchange (Waites et al., 2009). The demonstration that βSAP97–EGFP exchange rates are not altered by GluR1 cross-linking indicate that α- and βSAP97 do not shuttle GluR1 into and out of the spine as a complex but instead create docking sites that serve to transiently tether AMPARs in place, similar to what has been observed for PSD-95/TARP complexes (Bats et al., 2007). Furthermore, in agreement with several reports (Adesnik et al., 2005; Ashby et al., 2006; Bats et al., 2007), our electrophysiology & imaging revealed the majority of GluR1 in dendritic spines is at the cell surface, suggesting that α- and βSAP97 bind and retain these AMPAR subunits within distinct cell-surface domains.

We and our Stanford colleagues have examined whether βSAP97 regulates the synaptic localization and function of AMPARs. As with αSAP97, overexpression of βSAP97 increased the surface expression of endogenous and recombinant GluR1 within dendritic
spines. In addition, we observed a marked decrease in AMPAR-mediated synaptic transmission in these neurons that was consistent for both miniature and evoked currents. Furthermore, βSAP97 did not rescue the loss of AMPAR mEPSCs seen in neurons lacking PSD-95. These results clearly demonstrate that although βSAP97 strongly promotes the postsynaptic, cell-surface expression of GluR1-containing AMPARs, it binds and sequesters these receptors within an extrasynaptic domain, preventing their access to synaptic glutamate (Fig.3.5) (Waites et al., 2009).

In the present study, we aimed to evaluate the functional properties of α- and βSAP97 isoforms by studying their effects on AMPARs. Our data show that SAP97 isoforms specifically regulate AMPAR-mediated postsynaptic currents, miniature and evoked AMPAR EPSCs.
Figure 3-5 Model of how \( \alpha \)- and \( \beta \)SAP97 regulate the subsynaptic localization of GluR1-containing AMPARs.

A, Schematic diagram of an excitatory synapse at steady state. Cell-surface AMPARs are concentrated at the PSD through their interactions with PSD-95 but can laterally diffuse to extrasynaptic domains of the spine. B, Excitatory synapse expressing high levels of \( \alpha \) SAP97. This isoform localizes to the PSD, creating additional binding sites for surface AMPARs within this domain and restricting their lateral mobility. C, Excitatory synapse expressing high levels of \( \beta \)SAP97. This isoform localizes to extrasynaptic domains of the spine, creating new non-PSD binding sites for surface AMPARs and thereby inhibiting their diffusion into the PSD (Waites et al., 2009).
In summary, the combination of electrophysiology, live cell imaging and immunocytochemistry demonstrate that synaptic isoforms of SAP97 can directly regulate the levels, dynamics, and subsynaptic localization of GluR1-containing AMPARs. Moreover, as GluR1 has been implicated in the establishment of long-term potentiation, our work raise fundamental questions about whether different SAP97 isoforms affect GluR1 dynamics during synaptic plasticity.
Chapter 4. SAP97 directs NMDA receptor spine targeting and synaptic plasticity

Data in Chapter 4 have recently been published in The Journal of Physiology (Li et al., 2011). Chapter 4 is the original manuscript accepted for publication.

4.1. Abstract

SAP97 is a multidomain scaffold protein implicated in the forward trafficking and synaptic localization of NMDA- and AMPA-type glutamate receptors. Alternative splicing of SAP97 transcripts gives rise to palmitoylated αSAP97 and L27-domain containing βSAP97 isoforms that differentially regulate the subsynaptic localization of GluR1 subunits of AMPA receptors. Here, we examined whether SAP97 isoforms regulate the mechanisms underlying long-term potentiation (LTP) and depression (LTD) and find that both α- and β-forms of SAP97 impair LTP but enhance LTD via independent isoform-specific mechanisms. Live imaging of α- and βSAP97 revealed that the altered synaptic plasticity was not due to activity-dependent changes in SAP97 localization or exchange kinetics. However, by recording from pairs of synaptically coupled hippocampal neurons, we show that αSAP97 occludes LTP by enhancing the levels of postsynaptic AMPA receptors, while βSAP97 blocks LTP by reducing the synaptic localization of NMDA receptors. Examination of the surface pools of AMPA and NMDA receptors indicates that αSAP97 selectively regulates the synaptic pool of AMPA receptors, whereas βSAP97 regulates the extrasynaptic pools of both AMPA and NMDA receptors. Knockdown of βSAP97 increases the synaptic localization of both AMPA and NMDA receptors, showing that endogenous βSAP97 restricts glutamate receptor expression at excitatory synapses. This isoform-dependent differential regulation of synaptic versus extrasynaptic pools of glutamate receptors will determine how many receptors are available for the induction and the expression of synaptic plasticity. Our data support a model wherein SAP97 isoforms can regulate the ability of synapses to undergo plasticity by controlling the surface distribution of AMPA and NMDA receptors.
4.2. Introduction

Long-term potentiation (LTP) and depression (LTD) are two major forms of synaptic plasticity expressed in the hippocampus (Bliss & Collingridge, 1993; Malenka & Nicoll, 1999). The N-methyl-D-aspartate-type glutamate receptor (NMDAR) plays a key role in synaptic plasticity by acting as a coincidence detector to trigger the recruitment of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) into the synaptic membrane (Malenka & Nicoll, 1999; Malinow & Malenka, 2002; Citri & Malenka, 2008). Thus NMDARs are critical for the induction of synaptic plasticity while AMPARs are responsible for its expression.

The recruitment of receptors to synapses depends on their interaction with scaffold proteins at the postsynaptic density (PSD) (Montgomery et al., 2004; Specht & Triller, 2008). PSD-95 and SAP97 are central organizers of the PSD, simultaneously binding to receptors and to other scaffold or regulator proteins through PDZ, SH3 and GK interaction domains (Montgomery et al., 2004). Both PSD-95 and SAP97 bind the AMPAR subunit GluR1, although PSD-95 also requires the auxiliary protein stargazin (Leonard et al., 1998; Cai et al., 2002; Fukata et al., 2005). PSD-95 and SAP97 undergo alternative N-terminal splicing that gives rise to the α variants which contain a palmitoylation sequence, or β variants with an L27 protein interaction domain (Muller et al., 1995; Mori. et al., 1998; Chetkovich et al., 2002; McLaughlin et al., 2002; Schluter et al., 2006; Waites et al., 2009). The N-terminal sequences of SAP97 and PSD-95 control their dynamic properties (Chetkovich et al., 2002; Nakagawa et al., 2004; Waites et al., 2009). For example, the synaptic exchange rates of α- and βSAP97 differ widely, consistent with their association with structures localized within or outside the PSD, respectively (Waites et al., 2009). On many levels, αSAP97 mimics the properties of αPSD-95 (Nakagawa et al., 2004; Schluter et al., 2006; Waites et al., 2009). Nonetheless, given that αPSD-95 is the main expressed PSD-95 variant and βSAP97 the main form of SAP97, it appears that functional specialization has occurred between the two proteins (Chetkovich et al., 2002; Schluter et al., 2006; Waites et al., 2009). In contrast to αPSD-95, βSAP97 has multiple roles in the trafficking of receptor subunits, participating in the forward trafficking of AMPARs (Sans et al., 2001; Jeyifous et al., 2009), forming part of a multiprotein trafficking complex for Kir2 potassium channels (Leonoudakis et al., 2004), and trafficking NMDARs to synapses via Golgi outposts (Jeyifous et al., 2009).

Our previous work revealed that αSAP97 and βSAP97 differentially regulate the levels, dynamics and subsynaptic localization of AMPARs (Waites et al., 2009). Given the importance of AMPARs in the expression of synaptic plasticity, we examined how SAP97 isoforms could regulate synaptic plasticity mechanisms. By recording from synaptic
connections between individual hippocampal neurons, we demonstrate that α- and βSAP97 independently regulate LTP at hippocampal synapses by altering the synaptic and extrasynaptic localization of AMPARs and NMDARs respectively. Our data reveal that these isoform specific SAP97-induced changes in glutamate receptor localization have the potential to control the plasticity of hippocampal synapses.

4.3. Methods

4.3.1. Expression constructs

A restriction fragment (Nhe I / Xba I) containing the sequence of rat βSAP97 (splice variant I1b, I3 and I5), fused at its N-terminus with EGFP (Clontech, containing F64L/S65T) via the linker sequence SGLRSRAQASNS, was subcloned into the Xba I / Xba I backbone of the lentiviral vector pFUGW (Lois et al., 2002). This resulted in plasmid pFU-EG-rSAP97I3 (EMBL accession number AM710296; http://www.ebi.ac.uk/embl), for the expression of the fusion protein EGFP-βSAP97. Furthermore, we used expression constructs for αSAP97-EGFP (Schluter et al., 2006) and βSAP97-EGFP (Waites et al., 2009), also based on pFUGW and tagged at their C-terminus with EGFP. The shRNA construct used to specifically knock down the expression of SAP97 was targeted to the unique L27 domain of βSAP97. Both the βSAP97 shRNA and the scrambled control constructs have been described previously (Jeyifous et al., 2009). Sequence analysis reveals that αSAP97 has no unique domains: αSAP97 is identical to βSAP97 with the exception of the palmitoylation sequence, and αSAP97 shares this palmitoylation sequence with other MAGUKs (specifically αPSD93). Therefore it was not possible to create an shRNA that specifically targets αSAP97. Rescue of shRNA mediated knockdown was achieved by coexpressing an shRNA-resistant βSAP97-EGFP and shRNA-SAP97 (S12) in the pFU-rSAP97I3-EG vector. Silent mutations in codons encoding N-terminal amino acid residues 6,7,8,9 of βSAP97-EGFP was achieved by PCR with oligonucleotides containing the following sequence ATGCCGTTCCGAAGCAGAaCAaCAaAGA (bold: start codon; lower case: nucleotide changes). Note that this sequence is unique to βSAP97 and present near its L27 domain. The shRNA-SAP97 (S12) under the H1 polymerase III promoters was subcloned into the BsiW1-Pac1 sites upstream of the Ubiquitin promoter/βSAP97-EGFP expression cassette. Translocation studies (see Fig. 4.3) were conducted with YFP-tagged rat CaMKIIα (kind gift from J. Tsui) (Tsui et al., 2005).
4.3.2. Hippocampal neuron culture

Primary neuron cultures were prepared according to a modified Banker culture protocol (Banker & Goslin, 1998). Briefly, hippocampi from Sprague-Dawley rat embryos (E18-19) were dissociated in 0.05% trypsin (GIBCO, No. 25300) and plated on poly-L-lysine coated coverslips (Carolina Biological) at a density of 165 cells/mm². Coverslips were inverted after one hour, placed over a glial feeder layer and maintained at 37°C / 5% CO₂ in Neurobasal medium (GIBCO, No. 21103) containing 2 mM GlutaMAX (GIBCO, No. 35050) and B27 (GIBCO, No. 17504-044). Neuron cultures were infected with lentivirus expression constructs at DIV0-2 and used for experiments on DIV14-17. Alternatively, cultures were transfected by Ca₃(PO₄)₂ precipitation (on DIV7-9) or lipofectamine 2000 (Invitrogen) and used for experiments on DIV12-15. Most imaging experiments (localization studies, time-lapse imaging and FRAP) were done on lentivirus-infected neuron cultures, while the electrophysiological experiments were performed exclusively on transfected neurons. Control neurons were either untransfected neurons plated on the same coverslips as neurons transfected with α- or βSAP97, or neurons transfected with GFP alone. No significant difference in AMPAR or NMDAR EPSC amplitude was observed between untransfected and GFP-transfected neurons (Waites et al., 2009).

4.3.3. Hippocampal organotypic slice culture

Hippocampal slice cultures were prepared from P7 male rat pups as previously described (Montgomery et al., 2001). Briefly, 400 mm hippocampal sections were grown in MEM + 40% horse serum at 37°C on nitrocellulose membranes. At 3 DIV slices were moved to 34°C and maintained for 1-2 weeks. Slices were induced to express αSAP97-EGFP or EGFP-βSAP97 by lentiviral infection at 1 DIV. Paired whole cell recordings from CA3 pyramidal neurons were then performed at 7-11 DIV with the postsynaptic cell within the CA3 pair being GFP-positive indicative of expression of αSAP97-EGFP or EGFP-βSAP97.

4.3.4. Neuron transfection

A volume of 60 µl containing 2 µg DNA and 7.5 µl 2M CaCl₂ was added dropwise to 60 µl of 2 x HBS buffer (274 mM NaCl, 10 mM KCl, 1.4 mM Na₂HPO₄, 15 mM glucose, 42 mM HEPES, pH 7.1). After 20 min incubation in the dark, the precipitate was pipetted onto a coverslip of cultured neurons in 1 ml conditioned medium containing 50 µM APV and 10 µM CNQX. Neurons were incubated for 30 min at 37°C / 5% CO₂, rinsed three times with 2 ml pre-warmed HBSS and transferred back into culture dishes.
4.3.5. Lentivirus production

HEK cells were grown to confluence and cotransfected with pFUGW plasmids (10 µg) together with the helper plasmids VSVg (5 µg) and Δ8.9 (7.5 µg) in 1.5 ml Opti-MEM (GIBCO, No. 51985), using 60 µl lipofectamine 2000 (Invitrogen, No. 11668) according to the supplier’s protocol. Cells were transfected and maintained in neurobasal medium containing GlutaMAX and B27 at 32°C / 5% CO₂. The medium was exchanged once after 24 h and the virus was harvested in culture medium after ~55 h and stored at −80°C. Generally, lentivirus titers were in the range of 10⁸/ml, as estimated by the number of infected HEK cells expressing EGFP-fluorescence.

4.3.6. Imaging buffers

Buffers used for live imaging were based on Tyrode solution (120 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 25 mM HEPES pH 7.4, 30 mM glucose, sterile filtered). For control experiments, neurons were incubated in buffer solution containing 2 mM MgCl₂, 0.5 µM tetrodotoxin (TTX), 1 µM strychine, 50 µM APV and 10 µM CNQX, to fully block neuronal activity. To induce neuronal activity, we used conditions similar to those described by Ivanov and colleagues (Ivanov et al., 2006). For the direct activation of NMDARs, we applied 25 µM NMDA / 10 µM glycine in the presence of 0.5 µM TTX and 1 µM strychine (= cLTD treatment). Enhanced synaptic activity was achieved through the application of 20 µM bicuculline / 200 µM glycine in the presence of 1 µM strychine (= cLTP). For this application, prior incubations in control buffer were done in the absence of TTX. Similar treatments with NMDA or high concentrations of glycine have been shown to induce LTD or LTP in cultured hippocampal neurons, respectively (Lu et al., 2001).

4.3.7. Synaptic localization assay

Coverslips with hippocampal cultures expressing αSAP97-EGFP or EGFP-βSAP97 were transferred into Tyrode control solution containing Mg²⁺, TTX (not for subsequent bicuculline / glycine treatment), strychine, APV and CNQX at 37°C. After 5 min, the solution was exchanged with NMDA / glycine (cLTD) or with bicuculline / glycine containing solution (cLTP), or with the identical control solution (see imaging buffer compositions). After 10 min the neurons were fixed for 10 min at 37°C in 0.1 M sodium phosphate buffer pH 7.4, containing 4% paraformaldehyde and 1% sucrose. To correct for the expression levels of individual neurons, we quantified the synaptic accumulation of SAP97, i.e. the mean
fluorescence intensity of synaptic spines relative to the fluorescence in dendritic shafts, rather than the absolute levels of synaptic SAP97.

4.3.8. Live imaging

Time-lapse imaging was done on a scanning confocal microscope (custom-built by N. Ziv and S. Smith on a Zeiss Axiovert 100TV) with 488 nm and 514 nm lasers (Coherent; Sapphire 488-20CDRH and Compass 215M-20) and a 40x objective (1.3 NA; Zeiss Plan Neofluar), using OpenView software (by N. Ziv) for image acquisition. Coverslips with primary neurons were mounted in a perfusion chamber, maintained at 37°C and perfused with Tyrode-based solutions.

4.3.9. Fluorescence recovery after photobleaching (FRAP)

Synaptic puncta of EGFP-tagged SAP97 were bleached to ~20% of initial fluorescence intensity by multiple scanning passes (15-20 x) of a high intensity 488 nm laser beam. The fluorescence recovery was imaged for up to 30 min, initially every 30 seconds (14 frames) and then every 5 minutes (5 frames). Intensity values of bleached puncta at each time point were normalized to their fluorescence intensity prior to bleaching (I_t/I_pre) and to non-bleached control puncta (I_{nb,t}/I_{nb,pre}) in the same field of view. To calculate mean recovery traces the dynamic range of individual experiments was adjusted by setting the first value after photobleaching to zero. Thus, FRAP data were normalized according to the equations:

\[
F_{norm} = \frac{F_t - F_0}{F_{pre} - F_0}
\]

and

\[
F_{norm} = \frac{F_t}{I_{pre} \cdot I_{nb,t}}
\]

where F_{norm} is the normalized fluorescence intensity, F_t the intensity at time t, F_0 at time t = 0 and F_{pre} = 1 the intensity prior to photobleaching. The mean experimental data were fit with an exponential equation with two components, as described previously (Tsuriel et al., 2006):

\[
f(t) = a \cdot \left( 1 - e^{-\frac{t}{\tau_1}} \right) + (1 - a) \cdot \left( 1 - e^{-\frac{t}{\tau_2}} \right)
\]
where $\tau_1$ and $\tau_2$ are the time constants and $a$ and $(1-a)$ are the relative fractions of fluorescence in the two pools. The theoretical parameters were extracted by minimizing the sum of squared residuals, using a macro written in Excel (by N. Ziv). For time-lapse experiments, images were acquired every 3 minutes for up to 33 minutes (12 frames). Intensity values of synaptic SAP97 puncta were normalized to their fluorescence intensity prior to bleaching ($I/I_{pre}$), and expressed as mean intensity ± SEM.

4.3.10. Immunocytochemistry

Immunocytochemistry to detect $\alpha$-actinin (Sigma, 1:2000) was performed as previously described (Cheyne & Montgomery, 2008; Waites et al., 2009). Primary antibody binding was performed overnight at 4°C. Secondary antibody was performed for 1 hour at room temperature. Coverslips were washed and mounted onto slides (Vectashield) for imaging. Pyramidal neurons were identified by morphology and Z-stack (0.5 µm) images of dendrites were obtained on a Zeiss Axioskop with a CCD digital camera using a 63x oil objective. Image analysis was performed using Image J. Z-stacks were converted to 8-bit, merged into maximum projections and background subtracted to remove the diffuse protein expression within dendrites. Images in which staining was dim or the background was high were excluded from the analysis. Images were manually thresholded to select only puncta that were greater than 2-fold above image background. Puncta were analyzed using the Analyze Particles function in ImageJ so that the average puncta intensity and number of puncta could be determined. All data was normalized to parallel controls and presented as mean +/- SEM were $n$ is the number of fields analyzed. Statistical analysis was performed using student t-tests (one-tailed distribution, unequal variance).

4.3.11. Electrophysiology

Dual whole cell recordings were performed at DIV9-14 on primary dissociated hippocampal cultures and at DIV 7-11 on hippocampal organotypic slice cultures transfected with $\alpha$SAP97-EGFP or $\beta$SAP97-EGFP as previously described (Waites et al., 2009). Briefly, hippocampal cultures were transferred to a recording chamber on a Zeiss Axioskop 2FS and visualized under infrared differential interference contrast microscopy. Cultures were perfused at room temperature at a rate of 2 ml/min with artificial cerebrospinal fluid (ACSF, in mM: 119 NaCl, 2.5 KCl, 1.3 MgSO$_4$, 2.5 CaCl$_2$, 1 Na$_2$HPO$_4$, 26.2 NaHCO$_3$, 11 glucose). Presynaptic untransfected neurons were held in current clamp and induced to fire action potentials at 0.1 or 0.2 Hz by brief current injection (typically 20–50 pA for 20 ms).
Postsynaptic neurons (GFP-positive) were held in voltage clamp at -65 mV. Series resistance (Rs) was continuously monitored and recordings in which Rs varied by more than 20% were excluded from analysis. Internal solution consisted of (in mM): 120 K gluconate (presynaptic cell) or 120 Cs gluconate (postsynaptic cell), 40 HEPES, 5 MgCl₂, 0.3 NaGTP, 2 NaATP, 5 QX314 (postsynaptic cell only), pH 7.2 with KOH or CsOH. Evoked AMPAR and NMDAR-mediated EPSCs were measured as previously described (Montgomery et al., 2001; Montgomery & Madison, 2002; Montgomery et al., 2005; Waites et al., 2009). Isolated NMDAR EPSCs were measured at +40 mV in ACSF containing 10 µM NBQX. AMPAR and NMDAR EPSCs in response to presynaptic action potentials were collected at 0.1 or 0.2 Hz. LTP and LTD were induced 5 minutes after the initiation of the postsynaptic whole cell recording using either pharmacological stimulation of NMDARs as described above for live imaging or by pairing presynaptic action potentials with postsynaptic depolarization (Montgomery et al., 2001; Montgomery & Madison, 2002; Montgomery et al., 2005; Waites et al., 2009). To measure surface AMPAR and NMDAR-mediated currents, exogenous AMPA (1 mM) or NMDA (1 mM) was applied with a picospritzer (pressure 2 bar, pulse 200 ms). The micropipette was placed at a standard distance of 200 µm from the dendrites of α- or βSAP97-EGFP transfected neurons. The peak amplitudes of AMPAR- and NMDAR-mediated surface currents were measured by whole cell patch clamp as detailed above. Online data acquisition and offline analysis for all electrophysiology experiments was performed with pClamp (Clampex v9.2). Statistical significance of changes in AMPAR and NMDAR EPSC amplitudes was tested using the Students t-test with a level of significance set at p < 0.05.
4.4. Results

4.4.1. SAP97 isoforms regulate synaptic plasticity in hippocampal neurons

SAP97 is known to be critical for the trafficking of AMPA- and NMDA-type glutamate receptors to synapses (Sans et al., 2001; Jeyifous et al., 2009) and in directing the subsynaptic localization of AMPARs at excitatory synapses (Schluter et al., 2006; Jeyifous et al., 2009; Waites et al., 2009). Our previous data show that αSAP97 drives AMPARs into the PSD, whereas βSAP97 drives AMPARs to extrasynaptic sites (Waites et al., 2009). We therefore hypothesized that SAP97 isoforms are prime candidates to regulate changes in AMPAR expression that are known to occur with LTP and LTD. In order to determine the potential role of SAP97 isoforms in synaptic plasticity, we employed a combination of electrophysiology and live cell imaging in the dissociated hippocampal cell culture preparation. Dissociated cultures and pharmacological induction of plasticity were employed to enable us to visualize synaptic protein dynamics and to compare these data to the electrophysiological changes seen with the same induction protocols.

LTP and LTD were induced in dissociated hippocampal cultures by pharmacological stimulation of NMDARs. Using paired whole-cell recordings from pyramidal neurons, we measured the amplitude of evoked excitatory postsynaptic currents (EPSCs) at synaptic connections between individual hippocampal neurons before, during and after the induction of chemical LTP (cLTP; 20 µM bicuculline + 200 µM glycine, in the presence of 1 µM strychnine to avoid activation of glycine receptors) or chemical LTD (cLTD; 25 µM NMDA + 10 µM glycine + 1 µM strychnine, see methods).

In control hippocampal neurons, application of bicuculline and glycine induced an increase in the amplitude of the AMPAR EPSCs (Fig. 4.1A). Potentiation of the AMPAR-mediated currents developed gradually over the first 5 minutes after the pharmacological induction of plasticity and was sustained for the length of all paired recordings (n= 13 pairs). Twenty minutes after cLTP induction, the amplitude of the AMPAR EPSCs was increased to an average of 144.3 ± 16.4% of the baseline current amplitude (p < 0.01; n = 13 pairs). This cLTP was NMDAR-dependent, as application of the NMDAR antagonist AP5 (50 µM) during cLTP induction prevented the increase in AMPAR EPSC amplitude (average AMPAR EPSC amplitude was 94.7 ± 2.4% of baseline 20 minutes after cLTP + AP5, n = 5 pairs).
N-terminal SAP97 splice variants alter synaptic plasticity in hippocampal neurons.

A. AMPAR EPSC amplitudes measured from paired recordings between control hippocampal neurons or between neurons expressing α- or βSAP97-EGFP in the postsynaptic cell. cLTP was induced 5 minutes after attaining each paired recording by a 5 minute application of 20 µM bicuculline and 200 µM glycine + 1 µM strychnine. EPSC amplitudes are normalized to baseline, pre-cLTP AMPAR EPSC amplitudes. Top panels: representative postsynaptic traces, overlaid, in control, α- or βSAP97-EGFP expressing neurons before and after cLTP. One example of a presynaptic action potential trace is shown for each group.

B. AMPAR EPSC amplitudes measured from paired recordings between hippocampal neurons as described in part A. cLTD was induced by a 5 minute application of 25 µM NMDA and 10 µM glycine + 1 µM strychnine and paired recordings were held for up to 20 minutes post cLTD induction.

C. Surface AMPAR current amplitudes were measured by focal application of 1 mM AMPA in control, α- and βSAP97 expressing neurons immediately prior to and 20 minutes after the induction of cLTP or cLTD. Top: Sample traces of surface AMPAR-mediated currents measured in control, α- and βSAP97 expressing neurons. Bottom: Data were normalized to pre-cLTP or cLTD current amplitudes and are expressed as a percentage of the baseline surface AMPAR current.
We next examined whether the expression of two SAP97 isoforms, α- and βSAP97, altered this plasticity. These two N-terminal SAP97 isoforms were selected based on previous data showing that the palmitoylated αSAP97 recruits GluR1 containing AMPARs into the PSD and consequently increases basal synaptic transmission, while the L27 containing βSAP97 redirects GluR1 containing AMPARs into a peri-PSD compartment within dendritic spines, thus reducing basal synaptic transmission (Waites et al., 2009). Indeed, as expected the baseline levels of evoked AMPAR EPSC amplitudes were higher in neurons over-expressing αSAP97 and reduced in βSAP97-expressing neurons compared to control neurons, as reported previously (Waites et al., 2009). Average baseline AMPAR EPSC amplitude was 419.9 ± 54 pA in αSAP97-expressing neurons (n = 22 pairs) and 62.9 ± 16.1 pA in βSAP97-expressing neurons (n = 21 pairs). To assess the impact of α- and βSAP97 on the ability of excitatory synapses to undergo LTP, we measured EPSC amplitudes between pairs of hippocampal neurons in which the postsynaptic cells expressed EGFP-tagged αSAP97 or βSAP97. In contrast to untransfected neurons, we found that cLTP could not be induced in hippocampal neurons expressing αSAP97-EGFP or βSAP97-EGFP (Fig. 4.1A). In neurons expressing αSAP97, cLTP induction resulted in a decrease in the amplitude of AMPAR EPSCs. Twenty minutes after the induction of cLTP, the AMPAR EPSC amplitudes were 78.0 ± 13.9% of baseline control amplitudes (n = 10 pairs; Fig. 4.1A). Similarly, in neurons expressing βSAP97, cLTP induction resulted in a decrease in the amplitude of AMPAR EPSCs. Twenty minutes after the induction of cLTP, AMPAR EPSC amplitudes were decreased to 58.0 ± 13.7% of baseline current amplitudes (p < 0.01; n = 13 pairs). These data reveal that the overexpression of either SAP97 isoform impairs the induction and/or the expression of cLTP.

We next examined the ability of αSAP97 and βSAP97 to modulate long-term depression (LTD). In control neurons, the induction of cLTD produced an immediate and long-lasting depression of AMPAR-mediated EPSCs (Fig. 4.1B). AMPAR EPSC amplitudes were significantly decreased to 78.4 ± 14.8% of baseline current amplitudes (measured 20 minutes after cLTD induction; Fig. 4.1B; p < 0.01; n = 7 pairs). This cLTD was blocked by the NMDAR antagonist AP5, demonstrating its NMDAR dependence (average AMPAR EPSC amplitude was 106.3 ± 1.2% of baseline currents 20 minutes after cLTD + AP5; n = 5 pairs).

cLTD could also be induced in neurons expressing either αSAP97 or βSAP97 (Fig. 4.1B). As in control neurons, the amplitude of AMPAR EPSCs decreased immediately and this decrease was sustained in both αSAP97 and βSAP97 expressing hippocampal neurons for the length of the paired recordings. AMPAR EPSC amplitudes were 45.7 ± 11.1% of baseline current amplitudes in αSAP97 expressing neurons (n = 12 pairs; 20 minutes after cLTD induction). Similarly in βSAP97 expressing neurons, cLTD induction resulted in a
significant decrease in AMPAR EPSC amplitudes to 59.6 ± 8.3% (n = 8 pairs) of baseline current amplitudes. The level of depression in αSAP97 and βSAP97 expressing hippocampal neurons was significantly greater than the LTD expressed in control neurons (p < 0.01). These data show that neither SAP97 isoform prevents the pharmacological induction of LTD. However, as reported above, we observed a dramatic difference in the baseline levels of evoked AMPAR EPSC amplitudes in neurons over-expressing αSAP97 (higher) and βSAP97 (reduced) compared to control neurons (Fig. 4.1A), as reported previously (Waites et al., 2009). These differences support the general conclusion that N-terminal SAP97 isoforms differentially affect the synaptic levels of AMPARs, and also imply that the changes in establishing LTP and/or LTD could have different underlying mechanisms.

As differences in glutamate receptor trafficking have been suggested to occur in dissociated versus slice hippocampal preparations (Shi et al., 1999; Waites et al., 2009), we sought to determine whether αSAP97 and βSAP97 also altered the ability of synapses to express plasticity in hippocampal slices. We performed paired whole cell recordings from CA3 pyramidal cell pairs in which the postsynaptic neuron was expressing either EGFP-tagged α- or βSAP97. On average, baseline AMPAR-mediated EPSCs in control pyramidal cell pairs were 16.0 ± 2.3 pA, compared with 27.3 ± 8.4 pA and 14.0 ± 2.4 pA in pairs in which the postsynaptic neuron expressed αSAP97 or βSAP97 respectively. The increase in AMPAR EPSC amplitude in αSAP97-expressing neurons was significant (p < 0.01), demonstrating that αSAP97 exerts similar effects in dissociated and hippocampal slice in vitro preparations. The AMPAR EPSC amplitude in βSAP97-expressing neurons was not significantly different from controls. The smaller magnitude of the change in average AMPAR EPSC amplitude in the slice cultures may reflect the lower expression levels of α- and βSAP97 induced by lentiviral infection. However, we also noted a decrease in the probability of βSAP97-expressing CA3 pyramidal cell pairs having an evident AMPAR EPSC indicative of a synaptically connected pair (27.3% in βSAP97 expressing neurons, compared with 38.5% in control slices and 53.8% in αSAP97-expressing slices), suggesting that AMPAR-mediated currents may be decreased to undetectable levels or that CA3 pyramidal neuron connectivity is decreased in βSAP97-expressing neurons. We then induced LTP or LTD in each connected CA3 pyramidal cell pair by our electrical synaptic stimulation protocols described previously, resulting in robust LTP and LTD (Fig. 4.2; Montgomery et al., 2001; Montgomery and Madison, 2002; Montgomery et al., 2005). Twenty minutes after the LTP pairing protocol, average AMPAR EPSC amplitude was 210.2 ± 71.0% of baseline amplitude in control CA3 pyramidal cell pairs (n = 5 pairs, Fig. 4.2A). The induction of LTP between pyramidal cell pairs in hippocampal organotypic slices was also altered by the expression of
αSAP97 or βSAP97 but to a differing degree to that observed with cLTP in dissociated hippocampal cultures. Twenty minutes after the induction of LTP, average AMPAR EPSC amplitude was 126.4 ± 7.3% and 86.9 ± 5.6% of the baseline EPSC amplitude in pyramidal cell pairs in which the postsynaptic neuron expressed α- (n = 6 pairs) or βSAP97 (n = 5 pairs), respectively. Thus αSAP97 impaired and βSAP97 prevented the induction of LTP in hippocampal slices. Induction of LTD with 1 Hz presynaptic stimulation for 5 minutes induced robust LTD in control, αSAP97 and βSAP97-expressing neurons (Fig. 4.2B). AMPAR EPSC amplitudes were 47.1 ± 10.6%, 24.8 ± 6.3% and 29.0 ± 4.6% of baseline current amplitudes in control (n = 5 pairs), αSAP97 (n = 6 pairs) and βSAP97 (n = 6 pairs) expressing neurons, respectively. The magnitude of the LTD induced by 1 Hz stimulation was stronger compared to that induced by chemical stimulation in dissociated hippocampal cultures (Fig. 4.1B). Interestingly, the relative amounts of LTD expressed in control, α- and βSAP97-expressing neurons showed the same relationship in both forms of LTD. That is, the level of electrically-induced LTD in α- and βSAP97 expressing hippocampal neurons was significantly greater than that expressed in control neurons (p < 0.05 in both cases), as was observed in response to the induction of cLTD (Fig. 4.1B).
Figure 4-2 Plasticity induced by electrical stimulation in CA3 pyramidal cell pairs in organotypic hippocampal neurons expressing α- or βSAP97.

A. LTP was induced 5 minutes after attaining the postsynaptic whole cell recording by pairing presynaptic action potentials at 1 Hz with postsynaptic depolarization to 0 mV for 1 minute (arrow). In control neurons this induced a robust LTP (n = 5 pairs), however the expression of α- (n = 6) or βSAP97 (n = 5) in the postsynaptic cell of CA3 pyramidal cell pairs partially occluded or prevented the expression of LTP, respectively.

B. Induction of LTD by pairing presynaptic action potentials at 1 Hz with slight depolarization of the postsynaptic cell to -55 mV for 5 minutes induced robust LTD in control (n = 5 pairs), α- (n = 6) and βSAP97 (n = 6) expressing neurons. LTD was induced 5 minutes after each paired whole cell recording was attained. Amplitudes are expressed as a percentage of the baseline AMPAR current amplitude.
4.4.2. SAP97 isoforms differentially alter synaptic and extrasynaptic AMPAR pools

Our above analysis of αSAP97 and βSAP97-induced changes in LTP and LTD were measured as changes in synaptic AMPAR-mediated currents. Since we have previously shown that a significant proportion of AMPARs in βSAP97-expressing neurons are located in the extrasynaptic membrane, we examined whether the size of the surface pool of receptors was altered by the induction of synaptic plasticity in neurons expressing αSAP97 or βSAP97. Any changes observed in the size of the surface AMPAR currents could then be compared to the changes previously observed in synaptic AMPAR currents (Fig. 4.1A,B), providing insights into the redistribution of synaptic and extrasynaptic AMPAR pools during synaptic plasticity. In these experiments, we measured the amplitude of the surface AMPAR-mediated current in response to focal application of AMPA prior to and 20 minutes after cLTP or cLTD treatment (Fig. 4.1C). In control neurons, we found that the amplitude of the surface AMPAR-mediated current was not significantly altered by cLTP (average current amplitude was 90.6 ± 11.7% of baseline current amplitude 20 min after cLTP induction; n = 6; p = 0.08; Fig. 4.1C), despite the LTP-induced increase in the amplitude of the synaptic AMPAR-mediated currents (Fig. 4.1A). These data are consistent with the hypothesis that under control conditions, the increase in synaptic AMPAR current measured during LTP results from the recruitment of extrasynaptic AMPARs to synapses (Makino & Malinow, 2009).

We next examined how the surface pool of AMPARs was altered in neurons expressing αSAP97 or βSAP97. Consistent with βSAP97 inducing a higher level of total surface AMPARs localized to extrasynaptic sites (Waites et al., 2009), baseline surface current amplitudes were significantly higher in βSAP97-expressing neurons (Fig. 4.1C) (average current amplitude was 198.6 ± 45.3 pA in βSAP97 neurons versus 107.7 ± 25.5 pA in αSAP97 neurons; p < 0.001; n = 6), as previously reported (Waites et al., 2009). Following the pharmacological induction of LTP, we found that the amplitude of the surface AMPAR current was decreased in αSAP97-expressing neurons. Twenty minutes after the induction of LTP average current amplitude was 76.2 ± 13.5% of baseline currents (p < 0.05; n = 6; Fig. 4.1C), a decrease similar to that occurring in the synaptic pool (Fig. 4.1A). In contrast, the surface AMPAR-mediated current amplitude did not change with cLTP in βSAP97 neurons (95.7 ± 4.4% of baseline AMPAR current amplitude, p > 0.1, n = 6; Fig. 4.1C), despite the significant decrease in synaptic AMPARs with cLTP (Fig. 4.1A), suggesting that βSAP97 facilitates an increase in the size of the extrasynaptic pool of AMPARs with cLTP.
We also examined the amplitude of the surface AMPAR mediated current in response to the induction of cLTD. Here we found that cLTD resulted in a decrease in the amplitude of the surface AMPAR-mediated currents in control, αSAP97 and βSAP97-expressing neurons (control: 80.1 ± 11.3% of baseline AMPAR current amplitude; αSAP97: 64.6 ± 5.5% of baseline AMPAR current amplitude; βSAP97: 65.14 ± 13.4% of baseline AMPAR current amplitude; Fig. 4.1C, p < 0.01 in all cases). This decrease in the size of the total surface AMPAR mediated currents is similar to the changes seen in AMPAR-mediated synaptic currents in control, αSAP97 and βSAP97-expressing neurons (Fig. 4.1B), suggesting that with LTD expression both synaptic and extrasynaptic pools of AMPARs are changing in parallel and to a similar degree.

4.4.3. Synaptic plasticity does not alter the synaptic localization of SAP97 isoforms

Activity-dependent changes in synaptic strength have been directly linked to changes in the number of receptors localized in the PSD. If α- and βSAP97 are involved in directing AMPAR movement, then changes in α- and βSAP97 localization in response to synaptic activity may be responsible for the re-distribution of AMPARs during cLTP or cLTD. This concept is supported by several studies indicating that both the synaptic levels (Mauceri et al., 2004) and the protein dynamics (Nakagawa et al., 2004) of SAP97 may be regulated by synaptic activity. We designed a set of experiments to assess whether our cLTP or cLTD protocols caused a redistribution of SAP97 isoforms that could explain the observed changes of synaptic plasticity in α- and βSAP97-expressing neurons (Fig. 4.3). Initially, we examined the synaptic levels of EGFP-tagged α- and βSAP97 using time-lapse imaging. The intensity of synaptic SAP97 puncta were measured prior to, during and after the induction of cLTP or cLTD (Fig. 4.3A-C). No significant changes in the levels of α- or βSAP97 were detected either during or after the cLTP treatment (αSAP97: 99 ± 1.5% after 4 min of cLTP treatment and 99 ± 6.5% at 20 min after the treatment; βSAP97: 97 ± 2.0% after 4 min, 101 ± 5.4% after 20 min; Fig. 4.3B).

In contrast, there was a significant decrease in the fluorescence intensity of EGFP-βSAP97, and to a lesser extent of αSAP97-EGFP, during the induction of cLTD (αSAP97: 88 ± 6.1% after 4 min of cLTD treatment, p = 0.2; βSAP97: 63 ± 2.3%; p < 0.001, Mann-Whitnery U-test; Fig. 4.3A,C). These data imply that conditions that stimulate the reduction of synaptic AMPARs (i.e. cLTD) are perhaps causing the simultaneous removal of α- and βSAP97 from the spine. However, we also noted that this loss in fluorescence was reversible and was not associated with a long-term change in synaptic levels of αSAP97-
Figure 4-3 SAP97 localization is unchanged by cLTP or cLTD.

A-C. Lentivirus-infected neuronal cultures expressing αSAP97-EGFP or EGFP-βSAP97 were imaged for 5 min under baseline conditions, during 5 min application of bicuculline / glycine (cLTP treatment) or NMDA / glycine (cLTD) and after drug washout.

A. Sample images of EGFP-βSAP97 in spines (DIV15), imaged before (3 min timepoint shown; left image), during NMDA/glycine application (9 min; center image) and after washout (18 min timepoint; right image).

B. Quantification of time-lapse imaging data of αSAP97- (grey trace, n = 13 cells) and βSAP97-expressing neurons (black, n = 24) before, during and after cLTP induction, expressed as mean ± SEM. No significant changes in mean spine levels of SAP97 were detected at 3 versus 33 minute timepoints; Mann-Whitney U-test.

C. Application of NMDA and glycine was used to induce cLTD in neurons expressing αSAP97-EGFP (grey, n = 6) or EGFP-βSAP97 (black, n = 11).

D. Quantification of the spine/shaft ratio of αSAP97-EGFP fluorescence (DIV15; mean ± SEM; n ≥ 11 cells with 11 synaptic puncta per cell; right panel). αSAP97 enrichment did not change by either treatment (p = 0.8; Mann-Whitney U-test, two-tailed). E. Similarly, the cLTP and cLTD treatments produced no significant changes in the levels of βSAP97 in synaptic spines (n ≥ 14 random fields of view with ≥ 27 synaptic puncta per cell, analysis blind to condition; right panel).
EGFP or EGFP-βSAP97 (αSAP97: 103 ± 4.5% at 20 min after cLTD treatment; βSAP97: 101 ± 1.3%; Fig. 4.3C). Upon closer inspection, we noticed that the loss of fluorescence during the addition of NMDA/glycine occurred throughout the entire cell including dendrites (Fig. 4.3A) and soma (not shown), putting into doubt that an actual redistribution of the protein was taking place as had been previously proposed (Nakagawa et al., 2004). Instead, we hypothesized that the loss of EGFP-fluorescence was due to the acidification of the postsynaptic neuron during depolarization (Chesler, 2003) and thus due to quenching of the fluorophore (EGFP). In the presence of 25 µM NMDA, the intracellular pH of primary neurons decreases to about 6.5, recovering over a time course of minutes (Irwin et al., 1994). Furthermore, the fluorescence of EGFP is strongly pH-dependent, with a pKₐ ≈ 6 and a decrease by ΔpHi ≈ -0.5 leading to a reduction of fluorescence intensity of 20% (Kneen et al., 1998). These observations suggest that pH-dependent quenching of EGFP-based fluorophores is a confounding factor when using protocols that induce a prolonged depolarisation of neurons.

To test this quenching hypothesis, we repeated the time-lapse experiment with neurons expressing YFP-CaMKIIα (Fig. 4.4), which is known to be recruited to synapses by the activation of NMDARs (Shen & Meyer, 1999). Following the addition of NMDA/glycine to the imaging buffer, the YFP-fluorescence was quickly lost in all cellular compartments (Fig. 4.4A). Furthermore, the loss/quenching of YFP-CaMKIIα was more pronounced than that observed with EGFP-βSAP97, as expected for a fluorophore with a substantially higher pH sensitivity at physiological levels (Llopis et al., 1998). Moreover, after blocking excitatory neurotransmission with AP5 and CNQX, the fluorescence slowly recovered over a period of about 5 minutes (Fig. 4.4B). In a second experiment, neurons expressing YFP-CaMKIIα were stimulated with bicuculline/glycine or NMDA/glycine for five minutes and then immediately fixed (Fig. 4.4C). Here, we observed the translocation of YFP-CaMKIIα fluorescence into spines with both treatments, as reported previously (Shen & Meyer, 1999). These data clearly show that the apparent loss of fluorescence of YFP-CaMKIIα following the addition of NMDA/glycine is largely due to quenching and not to a redistribution of the protein. This quenching effect likely caused the reduction of YFP-βSAP97 fluorescence during NMDA application observed in an earlier study (Nakagawa et al., 2004). In our experiments the quenching by NMDA was more pronounced in neurons expressing EGFP-βSAP97 compared to αSAP97-EGFP (Fig. 4.3). The reason for this difference may result from α- and βSAP97 occupying different subsynaptic compartments that may be differentially affected by pH changes (Degiorgis et al., 2008; Waites et al., 2009).
Figure 4-4 cLTP and cLTD treatments cause YFP-CaMKII\(\alpha\) translocation into synaptic spines.

A. Time-lapse images of YFP-CaMKII\(\alpha\) translocation into spines. Sample images of dendritic segments (DIV15) acquired during a 3 min baseline conditions (left image; 1 min timepoint shown, see B), after application of 25 \(\mu\)M NMDA / 10 \(\mu\)M glycine (center image; 6 min), and after addition of 50 \(\mu\)M APV / 10 \(\mu\)M CNQX (right image; 11 min timepoint).

B. Quantification of data shown in A. Mean spine intensity levels were reduced during NMDA application (\(n = 6\) puncta). This quenching occurred in all cellular compartments and was not related to a redistribution of the YFP-CaMKII\(\alpha\). After addition of the glutamate receptor blockers APV and CNQX the fluorescence recovered over a period of 5 min. Note that the spine intensities after recovery exceed the levels prior to NMDA application as a result of CaMKII\(\alpha\) translocation (compare right and left image in A).

C. Neurons transfected with YFP-CaMKII\(\alpha\) were incubated with NMDA / glycine (cLTD) or bicuculline / glycine (cLTP) for 5 min before fixation and image acquisition (left panel)(DIV12). The enrichment of YFP-CaMKII\(\alpha\) in synapses was quantified as spine versus shaft fluorescence intensity (right panel) and showed that both treatments produced a translocation of CaMKII\(\alpha\) into dendritic spines (mean ± SEM; \(n = 3\) cells with \(\geq 17\) synaptic puncta per cell; control: shaft / spine ratio \(0.51 ± 0.06\); cLTD: \(0.93 ± 0.07\); cLTP: \(0.78 ± 0.12\)).
To evaluate the effect of cLTP and cLTD on the synaptic localization of EGFP-tagged α- and βSAP97 independently of quenching, we immediately fixed SAP97-expressing neurons after incubation in Tyrode buffer containing either bicuculline/glycine or NMDA/glycine (Fig. 4.3D,E). We found that neither cLTP nor cLTD induced significant changes in the localization of αSAP97 or βSAP97 (αSAP97: control spine/shaft levels 2.8 ± 0.2, LTD 2.9 ± 0.4, LTP 2.8 ± 0.4; βSAP97: control 2.5 ± 0.2, LTD 2.4 ± 0.1, LTP 2.5 ± 0.2; n ≥ 11 fields of view), indicating that the induction of synaptic plasticity does not change the steady-state synaptic levels of either of the two SAP97 isoforms.

4.4.4. Synaptic plasticity does not alter the exchange rate of SAP97

Our previous imaging studies revealed that the steady-state levels of synaptic scaffold proteins such as SAP97 are comprised of soluble, membrane- and cytoskeleton-associated pools (Waites et al., 2009). With regard to changes in synaptic strength, the most important is the size of the synaptic fraction of the scaffold proteins as this pool largely dictates the number and distribution of binding sites for neurotransmitter receptors within the PSD (Bredt & Nicoll, 2003; Bats et al., 2007; Waites et al., 2009). As such, shifts in the ratio of PSD-associated versus soluble pools of scaffold proteins could have a significant impact on receptor docking sites and synaptic strength. We therefore explored whether the induction of synaptic plasticity altered the exchange kinetics of EGFP-tagged α- or βSAP97 by performing fluorescence recovery after photobleaching (FRAP) experiments (Fig. 4.5).

Since the expression of long-term changes in synaptic strength are acquired over time, we evaluated whether the exchange kinetics of αSAP97-EGFP and EGFP-βSAP97 were changed 20 minutes after the pharmacological induction of synaptic plasticity (Fig. 4.5C,D). However, we could not detect any changes in the exchange kinetics of α- or βSAP97 after the induction of LTP or cLTD compared to control conditions. Therefore, the dynamics of α- and βSAP97 seem to be independent not only of the acute level of synaptic activity but were also unchanged by the chemical induction of LTP or LTD in these cultures, putting into doubt to what extent synaptic plasticity is actually involved in the regulation of SAP97 dynamics and vice versa. Taken together our data demonstrate that conditions that alter the synaptic strength in our system do not change the synaptic levels or protein dynamics of αSAP97 or βSAP97. This implies that the observed failure to induce LTP in neurons overexpressing α- or βSAP97 is not linked to changes in the dynamic properties of the scaffold proteins.
Figure 4-5 SAP97 dynamics are unchanged during induction and expression of cLTD or cLTP.

A,B. The exchange of synaptic αSAP97-EGFP and EGFP-βSAP97 was measured by FRAP during and after the application of NMDA / glycine (cLTD), bicuculline / glycine (cLTP) or under control conditions. Sample images of αSAP97-EGFP in lentivirus-infected hippocampal neurons (control condition) show the limited recovery of a bleached αSAP97 punctum (A, arrowhead) compared to the faster exchange of βSAP97 (B).

C,D. cLTD or cLTP were induced in lentivirus-infected neurons expressing αSAP97-EGFP or EGFP-βSAP97. Fluorescence recovery of bleached synaptic SAP97 was recorded with a delay of 20 min after drug washout.

C. Fluorescence recovery of αSAP97-EGFP after cLTD (dark grey trace, n = 22) or cLTP treatment (light grey, n = 13) did not vary from the exchange kinetics observed during blocked synaptic activity (control, black, n = 27).

D. Quantification of FRAP showed that the recovery of synaptic EGFP-βSAP97 was unchanged between control conditions (black trace, n = 20) and the neurons that had undergone cLTP or cLTD treatments followed by a 20 min delay (light and dark grey traces, n = 20 for both conditions).
4.4.5. SAP97 regulates the synaptic localization of NMDARs.

The activation of NMDARs is critical for the induction of many forms of synaptic plasticity. As LTP was absent in both αSAP97 and βSAP97-expressing neurons, we investigated whether SAP97 isoforms altered the synaptic expression of NMDARs. Using paired whole cell recordings, we first examined whether evoked NMDAR-mediated EPSCs between individual pairs of pyramidal neurons were altered by the overexpression of αSAP97 or βSAP97 in the postsynaptic neuron. Isolated synaptic NMDAR-mediated currents were measured between individual cultured hippocampal pyramidal neurons in response to presynaptic action potentials.

In control neurons, NMDAR-mediated EPSC amplitudes remained constant throughout the length of the paired recordings (minimum of 20 minutes; average NMDAR EPSC amplitude was 30.30 ± 1.28 pA, n=10 pairs; Fig. 4.6A). Interestingly, we found that αSAP97 and βSAP97 had differential effects on NMDAR-mediated EPSCs. In neurons expressing αSAP97, NMDAR EPSC amplitudes were not significantly different from control EPSCs (29.77 ± 0.78 pA, n = 12 pairs; p > 0.1). However, NMDAR EPSC amplitude was remarkably decreased in neurons expressing βSAP97 (9.41 ± 0.61 pA; n = 13 pairs; Fig. 4.6A), which was significantly decreased from both control and αSAP97-expressing neurons (p < 0.001 in both cases). This reduction in NMDAR-mediated EPSC amplitude in βSAP97-expressing neurons was not a result of a decrease in synapse number as we found no effect of either αSAP97 or βSAP97 on spine density in hippocampal neurons: in βSAP97-expressing neurons α-actinin density was 1.18 ± 0.13/µm, which was not significantly different from control neurons (1.01 ± 0.09/µm; p > 0.05) or αSAP97 expressing neurons (0.98 ± 0.16/µm; p > 0.05).

In order to determine whether the surface expression of functional NMDARs, comprising both synaptic and extrasynaptic pools, is altered in αSAP97 versus βSAP97-expressing neurons, we measured the surface NMDAR-mediated currents in hippocampal neurons by focal application of NMDA. Interestingly, despite the fact that βSAP97 decreases synaptic NMDARs, neurons expressing βSAP97 were more responsive to exogenous NMDA than those expressing αSAP97. NMDA-evoked current amplitudes were 112.47 ± 17.90 pA in βSAP97-expressing neurons, significantly higher than both αSAP97 expressing neurons and control neurons (alpha: 52.29 ± 5.38 pA, control: 60.00 ± 5.84 pA; both significantly different from βSAP97-expressing neurons, p < 0.001; Fig. 4.6C). Therefore, while βSAP97-expressing neurons have fewer functional NMDARs at the synapse for the detection of synaptically released glutamate, βSAP97 supports a significantly higher total surface expression of functional NMDARs.
Figure 4-6 βSAP97 is a negative regulator of synaptic NMDARs.

A. NMDAR EPSC amplitudes measured from paired recordings between pyramidal neurons in hippocampal cultures. NMDAR EPSCs were measured at +40 mV and paired recordings were maintained for a minimum of 20 minutes. Inset: representative traces of NMDAR EPSCs in control, α- and βSAP97 expressing neurons. Five postsynaptic traces are shown overlaid for each group, with 1 example of a presynaptic action potential.

B. Surface NMDAR-mediated currents evoked by focal application of NMDA to α- and βSAP97 expressing neurons. Right: Example traces of surface NMDAR-mediated currents shown overlaid for control, α- and βSAP97 expressing neurons.
4.4.6. Specific knockdown of βSAP97 increases synaptic expression of AMPA and NMDA receptors

To determine whether βSAP97 negatively controls the size of the synaptic pool of NMDARs in the endogenous situation, we used a short hairpin RNA (shRNA) to specifically downregulate the expression of endogenous βSAP97 in hippocampal neurons. Our previous studies have shown that this shRNA suppresses the expression of βSAP97 by more than 90% (Jeyifous et al., 2009). In contrast, previous studies have designed shRNA to the common GUK domain of SAP97 or utilized Dlgh1 mice lacking any functional SAP97 protein and therefore result in knockdown of all SAP97 isoforms (Howard et al., 2010). We found that specific knockdown of βSAP97 reverses the inhibitory effect previously observed on synaptic NMDAR-mediated EPSCs and results in a significant increase in synaptic NMDARs (Fig. 4.7A). Paired recordings revealed that the amplitude of NMDAR EPSCs in shRNA-expressing neurons was significantly higher than control neurons and in neurons expressing a scrambled shRNA [average NMDAR EPSC amplitude in shRNA expressing neurons was 115.44 ± 47.03 pA (n = 6 pairs), compared with 33.87 ± 8.15 pA in control neurons (n = 5 pairs) and 33.50 ± 5.95 pA in neurons expressing a scrambled shRNA (n = 5 pairs); p < 0.01]. Similarly, knockdown of βSAP97 reversed the previously observed decrease in synaptic AMPARs and also resulted in an increase in synaptic AMPARs [average AMPAR EPSC amplitude was 554.86 ± 287.42 pA in shRNA expressing neurons (n = 6 pairs), compared with 141.18 ± 40.10 pA in control neurons (n = 5 pairs) and 139.38 ± 21.35 pA in neurons expressing the scrambled shRNA (n = 5 pairs); p < 0.01; Fig. 4.7B]. The original effect of βSAP97, that is a decrease in both AMPAR and NMDAR EPSC amplitude compared with controls, could be rescued by expression of a modified βSAP97* that is resistant to knockdown (see methods; Fig. 4.7A,B. Together these data reveal that endogenous βSAP97 decreases the synaptic pools of NMDARs and AMPARs.

We also measured surface (i.e. synaptic + extrasynaptic) NMDAR- and AMPAR-mediated currents in βSAP97 shRNA-expressing neurons with exogenous application of NMDA or AMPA respectively. In response to exogenous application of NMDA, average surface NMDAR current amplitude was 136.98 ± 13.49 pA (n = 21 neurons) in βSAP97 shRNA-expressing neurons. In response to exogenous application of AMPA, average surface AMPAR current amplitude was 435.12 ± 59.57 pA (n = 24 neurons). Neither the surface NMDAR- or AMPAR-mediated current amplitudes in βSAP97 shRNA-expressing neurons were significantly different from the synaptic NMDAR or AMPAR EPSC amplitudes in βSAP97 shRNA-expressing neurons (NMDAR EPSC 115.44 ± 47.03 pA Fig. 4.7A, AMPAR EPSC 554.86 ± 287.42 pA Fig 4.7B; p > 0.05). Together these data are consistent
A. Knockdown of endogenous βSAP97 results in a significant increase in the amplitude of NMDAR mediated EPSCs that was not observed in the shRNA scrambled controls. Expression of the altered form βSAP97* that is resistant to knockdown rescued the phenotype observed in βSAP97-expressing neurons. Example traces of NMDAR-mediated EPSCs are shown for control (untransfected), shRNA βSAP97 and βSAP97* with a representative action potential. Scale bars: 50 pA/50 ms and 20 mV/20 ms.

B. Knockdown of endogenous βSAP97 also significantly increases the amplitude of AMPAR mediated EPSCs. Expression of the altered form βSAP97* that is resistant to knockdown rescued the phenotype observed in βSAP97-expressing neurons. In both A and B, average NMDAR and AMPAR EPSCs were measured from paired whole cell recordings in which the postsynaptic cells were either untransfected (controls), or expressing either shRNA βSAP97, scrambled shRNA or βSAP97* (see methods). Example traces of AMPAR-mediated EPSCs are shown for control (untransfected), shRNA βSAP97 and βSAP97* with a representative action potential. Scale bars: 100 pA/100 ms and 20 mV/20 ms.
with the redistribution of NMDA and AMPA receptors from extrasynaptic to synaptic sites in the absence of βSAP97.

**4.4.7. SAP97 isoforms alter the distribution of NMDARs during synaptic plasticity.**

The differential localization of NMDARs at synaptic and extrasynaptic sites in αSAP97 and βSAP97-expressing neurons respectively suggests that N-terminal isoforms of SAP97 may regulate the size of the NMDAR pools and contribute to a shift in NMDAR distribution between synaptic and extrasynaptic sites during synaptic plasticity. We therefore measured the amplitude of the surface NMDAR-mediated current in response to exogenous NMDA before and 20 minutes after the pharmacological induction of LTP or LTD (Fig. 4.8). We found that the surface pool of NMDARs was not altered by cLTP in control neurons or in neurons expressing αSAP97 (control: 123.6 ± 14.6%, n = 13; αSAP97: 116.5 ± 30.2% of baseline total surface NMDAR-mediated currents, n = 9; Fig. 4.8A). However, we observed that βSAP97 induced a significant increase in the surface pool of NMDARs after the pharmacological induction of LTP (202.6 ± 10.9% of baseline surface NMDAR current amplitude; n = 9, p < 0.001; Fig. 4.8A). To determine whether the βSAP97-induced increase in surface NMDARs was induced by changes in synaptic or extrasynaptic NMDARs, we performed paired whole cell recordings to measure isolated NMDAR-mediated EPSCs. The amplitudes of the NMDAR EPSCs were monitored before and after the pharmacological induction of LTP or LTD in control, α- and βSAP97-expressing neurons (Fig. 4.8). We found that cLTP induction caused a significant increase in the amplitude of the synaptic NMDAR EPSCs in control and αSAP97-expressing neurons (baseline current EPSC amplitude increased from 37.85 ± 6.4 pA to 58.5 ± 25.9 pA in control neurons 30 min post-cLTP, n = 6 pairs, and from 34.18 ± 8.27 pA to 55.57 ± 9.4 pA in αSAP97-expressing neurons 30 min post-cLTP, n = 8 pairs; Fig. 4.8B). In contrast, no significant change occurred in the amplitude of the NMDAR EPSCs in βSAP97-expressing neurons (EPSC amplitudes were 16.7 ± 2.1 pA and 13.4 ± 1.97 pA, prior to and 30 minutes after LTP, respectively, n = 10 pairs; Fig. 4.8B). Therefore, the LTP-induced increase in total surface NMDAR mediated currents in βSAP97-expressing neurons must result from an increase in the size of the extrasynaptic pool of NMDARs.

We also examined how the pharmacological induction of LTD altered the amplitude of surface NMDAR-mediated currents in control, α- and βSAP97-expressing neurons (Fig. 4.8C). Five minutes after cLTD induction, surface NMDAR-mediated currents were significantly decreased in control neurons (average total NMDAR-mediated surface current was 55.6 ± 13.1 % of baseline, p < 0.01, n = 6; not shown), but recovered to near baseline
Figure 4-8 Modulation of NMDARs by cLTP and cLTD in α- and βSAP97 expressing neurons.

A. Surface NMDAR-mediated current amplitudes evoked by focal application of NMDA before and 20 minutes after the induction of cLTP in control, α- and βSAP97 expressing neurons. Amplitudes are expressed as a percentage of the baseline NMDAR current amplitude.

B. NMDAR EPSC amplitudes measured from paired recordings between hippocampal neurons. cLTP was induced 5 minutes after attaining the postsynaptic whole cell recording. NMDAR EPSCs were measured at +40 mV and paired recordings were held for 40 minutes.

C. Surface NMDAR-mediated currents evoked by focal application of NMDA (1 mM) were measured before and 20 minutes after the induction of cLTD. Amplitudes are expressed as a percentage of the baseline NMDAR current amplitude.

D. NMDAR EPSC amplitudes measured from paired recordings between hippocampal neurons. cLTD was induced 5 minutes after the attainment of a postsynaptic whole cell recording and paired recordings were maintained for 30 minutes. All presynaptic neurons were untransfected, while the postsynaptic partner was either untransfected (control) or expressing α- or βSAP97-EGFP.
levels 20 minutes after the pharmacological induction of LTD (Fig. 4.8C: 87.8 ± 13.4 % of baseline current, p > 0.05, n = 6). Similarly, 5 minutes after cLTD induction, we found that surface NMDAR-mediated currents were significantly decreased in α- and βSAP97-expressing neurons (αSAP97: 56.2 ± 14.8 % baseline current, p < 0.01, n = 6; βSAP97: 48.8 ± 6.6 % baseline current, p < 0.01, n = 9; not shown). However in α- and βSAP97-expressing neurons, the depression of NMDAR-mediated currents did not recover, such that 20 minutes after cLTD induction total surface NMDAR-mediated currents were still significantly below baseline levels (αSAP97: 67.6 ± 14.6% and βSAP97: 44.8 ± 7.2 % of baseline currents, n = 6 and 9 respectively, p < 0.01 in both cases; Fig. 4.8C).

To determine whether these changes in NMDAR-mediated currents occurred at synaptic receptors, we performed paired whole cell recordings to measure the amplitude of isolated NMDAR EPSCs in control, α- and βSAP97-expressing neurons. We found that NMDAR EPSC amplitudes decreased significantly in control neurons, as previously described for electrically-evoked LTD (Montgomery & Madison, 2002; Montgomery et al., 2005). Similarly, in α- and βSAP97-expressing neurons, we found that NMDAR EPSC amplitudes decreased significantly and that this decrease persisted for the length of the paired recording (≥ 35 minutes; Fig. 4.8D; control: average baseline EPSC amplitude decreased from 31.51 ± 2.59 pA to 12.68 ± 1.66 pA at 25 min post-LTD, n = 7 pairs; αSAP97: from 31.42 ± 3.28 pA to 16.14 ± 3.94 pA at 25 min post-LTD, n = 6 pairs; βSAP97: from 14.93 ± 0.81 pA to 11.87 ± 0.95 pA at 25 min post-LTD, n = 7 pairs). Thus, in response to cLTD the size of the synaptic pool of NMDARs decreases in control, α- and βSAP97-expressing neurons, albeit to varying degrees. These decreases likely contribute to the observed reduction in surface NMDAR-mediated currents, but the differences we observed in the changes of these two pools of NMDARs again reflect the differential regulation of synaptic and extrasynaptic NMDARs by αSAP97 and βSAP97.

4.5. Discussion

In this study we have examined how N-terminal SAP97 isoforms regulate the induction and the expression of synaptic plasticity, and whether this occurs through activity-dependent regulation of the number of synaptic binding sites, or through the differential localization of NMDARs and AMPARs. The use of pharmacological paradigms to induce plasticity enabled us to directly examine how the same protocols influence synapse function via electrophysiology and protein dynamics using live cell imaging. Our data suggest that the capacity to induce and express synaptic plasticity is at least in part determined by how many
receptors are bound to either the PSD-dominant palmitoylated αSAP97, inducing increased clustering of AMPARs within the PSD, or to extrasynaptic L27 domain-containing βSAP97 that sequesters both AMPARs and NMDARs at extrasynaptic sites.

4.5.1. αSAP97 occludes synaptic potentiation by increasing synaptic AMPARs
α- and βSAP97 appear to prevent LTP via different mechanisms. As NMDAR-mediated synaptic currents were entirely normal in αSAP97-expressing neurons, these neurons possess the ability to induce LTP. We believe that the lack or the reduction of LTP is due to an increased steady-state localization of AMPARs in the PSD by αSAP97 (Figure 4.9) (Waites et al., 2009). Therefore LTP and αSAP97 overexpression appear to engage the same mechanisms to increase synaptic strength and the expression of LTP is occluded by αSAP97. As LTP was not accompanied by a change in αSAP97 exchange kinetics, this reveals that the number of αSAP97 AMPAR binding sites does not change, but that during LTP αSAP97 promotes the selective localization of AMPARs to the synaptic pool.

4.5.2. βSAP97 regulates plasticity by sequestering NMDARs outside of synapses
Our observation that βSAP97 prevents LTP, decreases synaptic NMDARs and increases surface NMDARs reveals that βSAP97 localizes NMDARs to extrasynaptic sites, as it does with AMPARs (Waites et al., 2009). This is supported by converse experiments where in the absence of βSAP97, synaptic AMPARs and NMDARs are increased whereas extrasynaptic receptors are decreased, indicating that a property of endogenous βSAP97 is to exclude these receptors from the synapse (Fig. 4.9). These data have led us to the conclusion that βSAP97 is a negative regulator of synaptic pools of NMDARs as well as AMPARs, consequently preventing the induction of LTP.
Figure 4-9 Model of SAP97 isoform–dependent regulation of synaptic glutamate receptor localization.

Our physiological data reveal that α- and β-isoforms of SAP97 play strikingly different roles in controlling the localization of AMPA and NMDA-type glutamate receptors at synaptic and extrasynaptic sites.

A. βSAP97 creates docking sites for AMPA and NMDA receptors out of the PSD, sequestering AMPA and NMDA receptors at extrasynaptic sites where they are unable to contribute to the induction and expression of LTP. The net result is a greater shift in receptor localization to the extrasynaptic sites. Both receptor types are able to freely diffuse away from the synaptic site however and be internalised in response to LTD.

B. αSAP97 creates docking sites for AMPA receptors in the PSD, shifting the localization of AMPA receptors into the synapse and increasing synaptic strength. No effect of αSAP97 on NMDAR localization or function was evident in our studies.
A previous model proposed that βSAP97 functions to either (a) facilitate the delivery of AMPARs to the extrasynaptic membrane from intracellular pools or (b) trigger the release of AMPARs that are retained in an extrasynaptic pool so they can be translocated to synapses (Rumbaugh et al., 2003; Schluter et al., 2006). While this model was in relation to AMPARs, our independent measurement of synaptic and surface NMDARs and AMPARs has enabled us to distinguish between these two possibilities. The observed high extrasynaptic levels of glutamate receptors are consistent with βSAP97 facilitating the delivery of AMPARs and NMDARs to the extrasynaptic membrane from intracellular pools. However, our data indicate that these receptors are sequestered outside of the synapse, preventing the induction of LTP and the movement of extrasynaptic AMPARs into synaptic sites.

Previous studies have produced conflicting data on the effect of βSAP97, and the deletion or knockdown of SAP97, on AMPAR and NMDAR EPSCs (Nakagawa et al., 2004; Schluter et al., 2006; Howard et al., 2010). A major reason for these discrepancies lies in the differing strategies employed to knockdown the expression of SAP97. In previous studies, only the effects of βSAP97 overexpression were examined. However, in the loss-of-function experiments the expression of all SAP97 isoforms were decreased by knockdown/knockout targeted to the GUK domain or exon 4 of SAP97 (Nakagawa et al., 2004; Howard et al., 2010). Therefore the combined synaptic effects of all SAP97 isoforms were reflected in these data. In the current study, we designed an shRNA to specifically target βSAP97, enabling us to exclusively determine the role of this dominant isoform in regulating the extrasynaptic pools of AMPA and NMDA receptors. Moreover, the extracellular stimulation employed in the previous studies may have activated both synaptic and extrasynaptic receptors, preventing the specific effect on extrasynaptic receptors being identified. Furthermore, the data discrepancies could also be caused by which I3/I4/I5 inserts are present in SAP97 (SAP97 with I3 and I5 used in the present study, not stated in other studies) and also the level and temporal expression profiles of βSAP97 having different effects on AMPAR and NMDAR currents (Howard et al., 2010). The fact that βSAP97 is also important in the forward trafficking of glutamate receptors (Sans et al., 2001; Jeyifous et al., 2009) adds yet another degree of complexity to the regulation of receptors by βSAP97. Our previously observed reduction in NR1 puncta intensity in βSAP97-deficient hippocampal neurons (Jeyifous et al., 2009) likely reflects a decrease in βSAP97-bound extrasynaptic surface receptors. However, by specifically decreasing βSAP97 expression in the present study, we further show that independent of its role in receptor trafficking, βSAP97 shifts the dynamic equilibrium of glutamate receptors from a synaptic to an extrasynaptic location.
Glutamate receptors have previously been proposed to be differentially regulated in hippocampal organotypic slices in comparison to dissociated hippocampal cultures, with GluR1-containing AMPARs being inserted upon stimulation in slices but under basal conditions in dissociated cultures (Shi et al., 1999; Waites et al., 2009). Our data support this conclusion in that we were able to induce LTP in αSAP97-expressing neurons in hippocampal slices but not in dissociated cultures, suggesting that a proportion of GluR1-containing receptors remained intracellular in the slice preparation and were inserted in response to LTP stimuli. As a result LTP was partially occluded by the expression of αSAP97, but was fully occluded in the dissociated culture system where GluR1 readily moves to the surface under basal conditions. Intriguingly, LTP was also blocked by the expression of βSAP97 in hippocampal organotypic slices, consistent with our conclusion from dissociated hippocampal cultures that βSAP97 prevents the movement of receptors into the synaptic space that is required for the expression of LTP.

The observed differences in synaptic AMPAR- and NMDAR-mediated synaptic currents induced by the expression of α- or β-SAP97 do not appear to result from differential effects on synapse number as we observed no effect of either αSAP97 or βSAP97 on spine density in hippocampal neurons or in surface GluR1 puncta (Waites et al., 2009). In addition, the significant decrease in the amplitude of the AMPAR- and NMDAR-mediated synaptic currents in βSAP97 expressing neurons, and the selective increase in the amplitude of the AMPAR- but not NMDAR-mediated synaptic currents in αSAP97-expressing neurons is not consistent with SAP97 isoforms increasing presynaptic glutamate release. Effects of SAP97 on presynaptic function have previously been observed in response to chronic overexpression of βSAP97 in vivo from E16 – P8, but presynaptic changes were not observed in immature neurons acutely expressing βSAP97 (Howard et al., 2010). Thus, our results likely reflect that N-terminal SAP97 isoforms regulate receptor targeting but not presynaptic function in early development.

α- and β-isoforms also exist for the MAGUK proteins PSD-95 and PSD-93. As the dominant SAP97 variant expressed at synapses is the β-isoform, and αPSD-95 the main form of PSD-95 (Chetkovich et al., 2002; Schluter et al., 2006), functional specialization may occur at the synapse as αPSD-95 stabilizes AMPARs in the PSD, while βSAP97 stabilizes receptors in the extrasynaptic pool. Similar to αSAP97, the α-isoform of PSD-95 has been shown to promote the synaptic clustering of AMPARs (Schluter et al., 2006), suggesting that there is some functional redundancy between α-isoforms with regards to LTP. The strong effects produced by βSAP97 expression or knockdown on AMPARs and NMDARs demonstrate that the L27 domain of βSAP97 bestows a unique and functionally important set of properties to this isoform. Our data also imply that the L27 domains in PSD-93 and
PSD-95 may similarly perform functions that modulate the ratios of synaptic versus extrasynaptic receptor complexes and thus may regulate synaptic plasticity in unexpected ways.

Following cLTP treatment, βSAP97 induced an increase in the extrasynaptic NMDAR pool, revealing that the size of extrasynaptic pools of receptors can also be regulated by plasticity. As there was no parallel increase in the synaptic NMDARs, these extrasynaptic receptors do not appear to merge with synaptic receptor pools as occurs during LTP in control neurons. Currently the role of the large extrasynaptic NMDAR pool in βSAP97-expressing neurons is unknown, but extrasynaptic signaling through NMDARs has been shown to be detrimental, activating cell death pathways and contributing to pathological conditions (Groc et al., 2009; Milnerwood et al., 2010). Therefore βSAP97 may play a role in the altered NMDAR signaling and trafficking observed in conditions such as Alzheimer’s and Huntington’s disease, schizophrenia and addiction (Marcello et al., 2007; Groc et al., 2009; Milnerwood et al., 2010).

As LTD could still be induced in α- and βSAP97-expressing neurons, N-terminal SAP97 isoforms allow receptor movement away from synaptic sites. In control, α- and βSAP97-expressing neurons, depression of the amplitude of AMPAR- and NMDAR-mediated currents was observed in the synaptic and the surface pools, reflecting a reduction in the size of both the synaptic and extrasynaptic receptor pools during LTD. This is consistent with the movement of AMPARs and NMDARs to membrane sites lateral to the PSD and their subsequent internalization at extrasynaptic endocytic zones to drive synapses to a depressed state (Blanpied et al., 2002; Montgomery & Madison, 2002; Ashby et al., 2004; Racz et al., 2004; Montgomery et al., 2005; Petrini et al., 2009). The internalization of NMDARs was most pronounced in neurons expressing βSAP97, where we observed a strong reduction of the surface receptor levels after LTD. This may result from the high baseline levels of extrasynaptic surface NMDARs in these neurons that facilitate NMDAR-dependent cLTD induction and receptor movement to extrasynaptic endocytic sites.

### 4.5.3. Synaptic plasticity does not affect the dynamics or distribution of N-terminal SAP97 isoforms

Previous studies have shown contradictory results with respect to the activity-dependence of SAP97 localization and dynamics. Treatment of hippocampal neurons with NMDA has been suggested to recruit βSAP97 to the synapse by CaMKII phosphorylation at Ser39 (Mauceri et al., 2004), or conversely to lead to the dissipation of βSAP97 from
synapses (Nakagawa et al., 2004). In contrast, we have shown that neither α- nor βSAP97 localization or dynamics were altered by the chemical LTP or LTD protocols. These differences could be due to fluorescence quenching described earlier, the βSAP97 isoform used in these studies (βSAP97 containing the I3/I5 inserts in our experiments; not stated in the other reports), or the level of SAP97 overexpression in comparison to the low levels expressed in the current study by lentiviral infection. Another possibility is the differences in concentration and length of treatment with NMDA utilized in the previous studies (50 µM for 15 minutes or 3 µM for 20 minutes). It is not known what, if any, changes in synaptic strength were induced by these protocols. In contrast, the protocols used in the current study were utilized in both imaging and electrophysiology experiments, and were shown in control neurons to induce LTP or LTD and to cause the translocation of CaMKII to synapses.

Ultimately, we aimed to determine whether there is a relationship between SAP97 dynamics and synaptic plasticity? We found little evidence that either α or βSAP97 alter their distribution or exchange kinetics in response to changes in synaptic activity. This implies that they exert their effects on synaptic function by creating surface glutamate receptor docking sites within the PSD and extrasynaptic space. At present, we find no evidence to support a model wherein either α- or βSAP97 perform a chaperone function for these receptors. This is not to say that receptor binding to these scaffold proteins is not regulated. Our cLTD data clearly demonstrate that surface AMPA and NMDA receptors can uncouple from the cytoskeletal matrix and be internalized. Such a model is consistent with studies showing that both AMPA and NMDA receptor binding to scaffold proteins is regulated by post-translational modifications such as phosphorylation and that SAP97 isoforms use their multidomain structure to create tertiary complexes with both kinase and phosphatases (AKAP/calcineurin) capable of responding to changes in synaptic signalling (Colledge et al., 2000; Tavalin et al., 2002; Montgomery et al., 2004; Dell'Acqua et al., 2006; Shepherd & Huganir, 2007). Taken together, our data show that α- and βSAP97 do not regulate synaptic plasticity via activity-dependent changes in their synaptic localization or dynamics, but rather are a platform for regulating the tethering of receptors in synaptic or extrasynaptic compartments.
4.6. Conclusions

We have performed parallel functional and imaging studies that revealed that N-terminal α and β splice variants of SAP97 differentially regulate the synaptic localization of AMPARs and NMDARs, resulting in major changes in the ability of neurons to express synaptic plasticity. Our data show that αSAP97 is important for localizing AMPARs in the PSD and increases the synaptic strength, but that these receptors are readily removed from the synapse in response to LTD. In contrast, βSAP97 appears to be a negative regulator of synaptic potentiation, favoring a shift towards synaptic depression by sequestering both AMPARs and NMDARs at extrasynaptic sites. As a result βSAP97 can block and/or modulate both the induction and the expression of synaptic plasticity. Together these data reveal that synaptic SAP97 isoforms can play functionally distinct roles in regulating glutamate receptor levels at synapses and as such influence synaptic plasticity mechanisms.
Chapter 5. The role of βSAP97-I2 isoform in regulating AMPAR- and NMDAR currents in hippocampal neurons

5.1. Introduction

The βSAP97-I2 isoform is one of multiple alternatively spliced insertion domain isoforms of SAP97. In SAP97, the hook region between the SH3 and GUK domains is a site of alternative splicing. Alternative splicing patterns in this region with the insertion of major splice forms, with either insertion 2 (I2; 34 nucleotides) or insertion 3 (I3; 100 nucleotides) have been reported (Lue et al., 1994; Mori. et al., 1998) and four short sequence elements (I2, I3, I4 and I5), in various combinations, have also been found to be inserted at the site between the SH3 and GUK domains (Lue et al., 1994; Muller et al., 1995; Rumbaugh et al., 2003). In hippocampal neurons, the SAP97-I2 isoform is expressed in the neuronal soma and dendrites, whereas the SAP97-I3 isoform is expressed mainly in dendritic spines (Valeria Albornoz et al., 2008). The major splice isoforms of SAP97 are often studied by investigators, with most focussing their studies on the insert I3, as βSAP97 containing the I3 insert has been shown to be essential for the synaptic localization of SAP97 (Rumbaugh et al., 2003). Ideally the I2- and I3-insert containing SAP97 isoforms should be studied in parallel. Similar to the I3 insert, SAP97-I2 has extensive and different expressing patterns in multiple important organs. For example, the I2 and I3 transcripts are expressed in the thymus, spleen, kidney and pancreas almost equivalently, but the I3 transcript appears to more predominant in the small intestine, stomach, uterus, placenta, and liver. Furthermore, the I2 transcript in skeletal muscle and the heart is hardly detectable, indicating that these two tissues express the transcript I2 significantly less abundantly. To date, most functions of the insert I2 of SAP97 in these organs are not fully understood. Thus, it is necessary to investigate the functional roles of the insert I2 of SAP97 in these tissues. However, most work investigating the function of the insert I2 in SAP97 isoforms is just in its initial stages.

Although the function of the hook domain insert is not generally known, the I3 insert of SAP97 has been found to bind to the actin/spectrin binding protein, protein 4.1 via I3 and sequences situated between PDZ1 and PDZ2. However, the SAP97I2 isoform does not bind to the membrane cytoskeletal protein 4.1 (Wu et al., 2000). According to previous work, binding to protein 4.1 is important to the function of SAP97, as dramatic loss in spine localization is observed when deleting the hook region in the SAP97 mutant (ΔI3). This suggests that binding between protein 4.1 and SAP97 is critical for synaptic targeting (Godreau et al., 2003). The SAP97 hook domain isoforms differ by alternatively spliced
insertion domains that regulate protein localization and oligomerization. SAP97 with alternatively spliced inserts I2 and I3 differentially affect GKAP binding (Godreau et al., 2003). However it is unknown whether the I2 insertion affects the recruitment of SAP97 to synapses and further affects synaptic transmission. The functional role of insert I2 in other tissues implies that it is likely to have an important function in brain. For example, SAP97-I2 can regulate the potassium channel in cardiac muscle. The cardiac SAP97 isoforms containing I2- and I3 inserts differently affect the currents of potassium Kv1.5 channels, with SAP97-I2 domain instead of the I3 domain increasing the current amplitude (Rumbaugh et al., 2003). In cells expressing I3, Kv1.5 channels were organized in plaque-like structures at the plasma membrane level, whereas intracellular aggregates of channels predominated with the I2 isoform. The two cardiac SAP97 insert isoforms have different effects on the hKv1.5 current, depending on their capacity to form channel clusters (Rumbaugh et al., 2003). These results raise the possibility that SAP97-I2 has the potential ability to regulate channel function in the brain.

To date, there is little evidence to support SAP97-I2 being an important candidate in regulating synaptic function according to the subcellular distribution of SAP97-I2 in neurons. The distribution of SAP97-I2 in neurons was described as diffuse throughout the soma and dendrites, not specifically concentrated in spines (Hanada et al., 2003). Furthermore, the I2 construct failed to increase the size of spines in high-density cortical neurons (Rumbaugh et al., 2003). Immunocytochemistry studies using I2- and I3-specific antibodies detected the I2 insert-specific antigen in the nucleus and the I3 insert-specific antigen in the plasma membrane, subsequently implicating the I2 insert in nuclear targeting function (Waites et al., 2009; Li et al., 2011). Moreover, overexpression of SAP97-I3 in neurons enhances the size of dendritic spines, the number of surface AMPA receptors, and AMPAR- and NMDAR-mediated EPSCs, whereas the I2 isoform did not induce any of these changes (Waites et al., 2009; Li et al., 2011). Until now, due to limited direct electrophysiological evidence, it is difficult to confirm whether the insert I2 has a central role in the brain. The N-terminal domain of SAP97 together with the I2 insert might endow SAP97-I2 important roles in the brain, with one report indicating that the deletion of the I3 region only reduced the efficiency of membrane targeting (Wu et al 1998), but the N terminus alone was sufficient to mediate targeting of SAP97 to sites of cell–cell contact (Doerks, 2000; Roh et al., 2002). The N terminus of SAP97 is involved in neuronal targeting, therefore the N terminus with the I2 insert might be sufficient to mediate SAP97 targeting to synapses to alter synaptic transmission and/or plasticity.

Together, the above information shows that the functional role of SAP97-I2 is not fully understood. Specifically, we do not know the role of SAP97 I2 in regulating synaptic function and neural transmission, for example, synaptic plasticity. In this study, we have examined
the βSAP97I2 in regulating synaptic function, especially in affecting glutamate receptors, and AMPARs and NMDARs in hippocampal neurons.

5.2. Results

Firstly, we examined the cellular distribution of βSAP97-I2 in hippocampal neurons transfected with EGFP-βSAP97-I2 (Fig. 5.1). βSAP97-I2 had an expression pattern similar to that of βSAP97 I3–EGFP, with diffuse somatodendritic labelling and punctate labelling of spines (Fig. 5.1).

5.2.1. βSAP97-I2 regulates synaptic transmission and plasticity in cultured hippocampal neurons

SAP97 is vital for the trafficking of NMDA- and AMPA-type glutamate receptors to synapses and in controlling the subsynaptic localization of AMPA and NMDA receptors at excitatory synapses (Nakagawa et al., 2004). Our previous data show that SAP97-I3 isoforms drive AMPARs into the PSD or to extrasynaptic sites (Jeyifous et al., 2009). We therefore hypothesized that βSAP97-I2 is also a candidate to regulate changes in AMPAR expression in synaptic plasticity. In order to determine the potential role of SAP97 I2 in synaptic plasticity, we performed a combination of electrophysiology and immunohistochemistry in the dissociated hippocampal cell culture preparation. Dissociated cultures and pharmacological induction of plasticity were utilised to enable us to visualize synaptic protein localisation and to compare these data to the electrophysiological changes seen with the same induction protocols. Long term potentiation and Long term depression were induced in hippocampal neurons by pharmacological stimulation of NMDA receptors as described in Chapter 4. Using paired whole-cell recordings from pyramidal neurons, we measured the amplitude of evoked excitatory postsynaptic currents (EPSCs) at synaptic connections between individual hippocampal neurons before, during and after the induction of chemical LTP (cLTP; 20 µM bicuculline + 200 µM glycine and 1 µM strychnine to avoid activation of glycine receptors) or chemical LTD (cLTD; 25 µM NMDA + 10 µM glycine + 1 µM strychnine).

Average baseline AMPAR EPSC amplitude was 236.9 ± 51 pA in control neurons (n = 16 pairs) and 72.9 ± 11.3 pA in βSAP97-I2 expressing neurons (n = 13 pairs). We next
Figure 5-1 Overexpression of βSAP97-I2 EGFP in hippocampal neuron.

βSAP97-I2 EGFP was expressed in cultured hippocampal neurons at 12 days in vitro (DIV12) with diffuse somatodendritic labeling and punctate labeling of spines. Arrows represent soma and spines. Scale bar: 20µm.
determined whether βSAP97-I2 alters the ability of neurons to undergo LTP. In control hippocampal neurons, application of bicuculline and glycine induced an increase in evoked AMPAR excitatory postsynaptic currents (EPSCs) (Fig. 5.2). Potentiation of the AMPAR-mediated EPSC amplitude developed gradually over the first five minutes after the pharmacological induction of plasticity and was sustained for the length of all paired recordings (n= 16 pairs). Twenty minutes after cLTP induction, the amplitude of the AMPAR EPSCs was increased to an average of 141.1 ± 13.2% of the baseline current amplitude (p < 0.01; n = 16 pairs).

We next examined whether the expression of βSAP97-I2 isoform altered this synaptic plasticity. We measured EPSC amplitudes between pairs of hippocampal neurons in which the postsynaptic cells expressed EGFP-tagged βSAP97-I2 isoform. In contrast to untransfected control neurons, we found that cLTP could not be induced in hippocampal neurons expressing βSAP97 I2-EGFP (Fig.5. 2). In neurons expressing βSAP97-I2, cLTP induction resulted in a decrease in the amplitude of AMPAR EPSCs. Twenty minutes after the induction of cLTP, the AMPAR EPSC amplitudes were 76 ± 11.3% of baseline control amplitudes (p < 0.01; n = 13 pairs; Fig. 5.2). These data reveal that the overexpression of βSAP97-I2 isoform impairs the induction and/or the expression of cLTP.

We next examined the ability of SAP97-I2 to modulate long-term depression (LTD). In control neurons, the induction of cLTD produced an immediate and long-lasting depression of AMPAR-mediated EPSCs (Fig.5.3). AMPAR EPSC amplitudes were significantly decreased to 76.3 ± 11.2% of baseline current amplitudes (measured 20 minutes after cLTD induction in control cells; Fig. 5.3; p < 0.01; n = 11 pairs). cLTD could also be induced in neurons expressing the βSAP97-I2 isoform (Fig. 5.3). Similar to that of the results found in control neurons, the amplitude of AMPAR EPSCs decreased immediately and this decrease was sustained in βSAP97-I2 expressing hippocampal neurons for the length of the paired recordings. AMPAR EPSC amplitudes were 48.3 ± 15.1% of baseline current amplitudes in βSAP97I2 expressing neurons (n = 6 pairs; 20 minutes after cLTD induction). The level of depression in βSAP97-I2 expressing hippocampal neurons was significantly greater than the LTD expressed in control neurons (p < 0.01). These data show that the βSAP97 I2 isoform does not prevent the pharmacological induction of LTD. However, we observed a dramatic difference in the baseline levels of evoked AMPAR EPSC amplitudes in neurons over-expressing βSAP97-I2 (reduced) compared to control neurons (higher). Therefore, βSAP97-I2 isoform allows receptor movement away from synaptic sites.
Figure 5-2 cLTP can not be induced in βSAP97-I2 overexpressing hippocampal neurons.

AMPAR EPSC amplitudes measured from paired recordings between hippocampal neurons. cLTP was induced 5 minutes after attaining the postsynaptic whole cell recording. All presynaptic neurons were untransfected, while the postsynaptic partner was either untransfected (control) or expressed βSAP97-I2-EGFP.
Figure 5-3 cLTD was induced in both βSAP97-I2 expressing and control hippocampal neurons.

AMPAR EPSC amplitudes measured from paired recordings between hippocampal neurons. cLTD was induced 5 minutes after attaining the postsynaptic whole cell recording. All presynaptic neurons were untransfected, while the postsynaptic partner was either untransfected (control) or expressed βSAP97-I2-EGFP.
The above analysis of βSAP97 I2-induced changes in LTP and LTD were measured as changes in synaptic AMPAR-mediated currents. Since we have previously shown that another insertion of SAP97, βSAP97 containing the I3 isoform, can remarkably regulate the location of AMPARs in the extrasynaptic membrane, we examined whether the size of the surface pool of receptors was altered in neurons expressing the βSAP97 I2 isoform. Any changes observed in the size of the surface AMPAR currents could then be compared to the changes previously observed in synaptic AMPAR currents, providing insights into the redistribution of synaptic and extrasynaptic AMPAR pools. In these experiments, we measured the amplitude of the surface AMPAR-mediated current in response to focal application of 1mM AMPA as described in Chapter 4. Similar to βSAP97-I3 inducing a higher level of total surface AMPARs localized to extrasynaptic sites, the baseline surface current amplitudes were significantly higher in SAP97 I2-expressing neurons compared to control and untransfected neurons (average current amplitude was 308.6 ± 36.3 pA in βSAP97-I2 neurons versus 137.5 ± 21.3 pA in control neurons; p < 0.001; n = 6) (Fig. 5.4).

The activation of NMDARs is pivotal for the induction of many forms of synaptic plasticity. As LTP was absent in βSAP97 I2-expressing neurons, we investigated whether the βSAP97-I2 isoform altered the synaptic expression of NMDARs. Using paired whole cell recordings, we first examined whether evoked NMDAR-mediated EPSCs between individual pairs of pyramidal neurons were altered by the overexpression of βSAP97 I2 in the postsynaptic neuron. Isolated synaptic NMDAR-mediated currents were measured between individual cultured hippocampal pyramidal neurons in response to presynaptic action potentials. In control neurons, after pharmacologically isolating NMDAR currents by adding of 10 µM NBQX to artificial cerebrospinal fluid (ACSF), we found that NMDAR-mediated EPSC amplitudes remained constant throughout the length of the paired recordings (minimum of 20 minutes; average NMDAR EPSC amplitude was 33.26 ± 1.32 pA, n=8 pairs; Fig. 5.5). However, in neurons expressing SAP97-I2, the NMDAR EPSC amplitude was remarkably decreased (5.67 ± 0.83 pA; n = 10 pairs; Fig.5.5), which was significantly decreased from control neurons (p < 0.001).

To determine whether the small amplitude synaptic NMDARs-mediated EPSCs observed in βSAP97 I2-expressing neurons could be increased with LTP, we performed paired whole cell recordings to measure isolated NMDAR-mediated EPSCs. The amplitudes of the NMDAR EPSCs were then monitored before and after the pharmacological induction of LTP in control and βSAP97 I2-expressing neurons (Fig. 5.6).
The amplitude of the surface AMPAR-mediated current was measured in response to focal application of 1mM AMPA in either untransfected (control) or expressed βSAP97-I2-EGFP hippocampal neurons, * p<0.05.
Figure 5-5. Synaptic NMDAR-mediated currents in βSAP97-I2 expressing neurons are significantly smaller than that of control neuron currents.

Synaptic NMDAR EPSC amplitudes measured from paired recordings between hippocampal neurons. All presynaptic neurons were untransfected, while the postsynaptic partner was either untransfected (control) or expressed βSAP97-I2-EGFP. NMDAR EPSC amplitudes measured from paired recordings between pyramidal neurons in hippocampal cultures. NMDAR EPSCs were measured at +40 mV in presence of 10uM NBQX and paired recordings were maintained for a minimum of 30 minutes.
Figure 5-6 cLTP of synaptic NMDAR-mediated currents can not be induced in SAP97-I2 expressing neurons.

No cLTP of synaptic NMDAR-mediated currents in βSAP97-I2 expressing neurons. In contrast, cLTP of synaptic NMDAR-mediated currents can be induced in control. Synaptic NMDAR EPSC amplitudes measured from paired recordings between hippocampal neurons. All presynaptic neurons were untransfected, while the postsynaptic partner was either untransfected (control) or expressed βSAP97-I2-EGFP.
We found that cLTP induction caused a significant increase in the amplitude of the synaptic NMDAR EPSCs in control neurons (baseline current EPSC amplitude increased from 38.13 ± 3.6 pA to 62.3 ± 21.5 pA in control neurons 30 min post-cLTP, n = 8 pairs, Fig. 5.6). In contrast, no significant change occurred in the amplitude of the NMDAR EPSCs in βSAP97 I2-expressing neurons (EPSC amplitudes were 14.9 ± 3.1 pA and 13.7 ± 2.15 pA, prior to and 30 minutes after LTP, respectively, n = 7 pairs, p>0.05; Fig. 5.6).

In order to determine whether the surface expression of functional NMDARs, comprising both synaptic and extrasynaptic pools, is altered in βSAP97 I2 versus control neurons, we measured the surface NMDAR-mediated currents in hippocampal neurons by focal application of 1mM NMDA as described in Chapter 4. Interestingly, despite the fact that βSAP97 I2 decreases synaptic NMDARs, neurons expressing βSAP97 I2 were more responsive to exogenous NMDA than those untransfected control cells. NMDA-evoked current amplitudes were 136.43 ± 15.68 pA in βSAP97 I2-expressing neurons, which is significantly higher than that of control neurons (62.17 ± 4.38 pA; significantly different from βSAP97 I2-expressing neurons, n=12, p < 0.001; Fig. 5.7). Therefore, while βSAP97 I2-expressing neurons have fewer functional NMDARs at the synapse for the detection of synaptically released glutamate, βSAP97 I2 supports a significantly higher total surface expression of functional NMDARs.

This result is consistent with our immunochemistry data that showed the intensity of NR1 surface immunolabelling in βSAP97-I2 overexpressing neurons is significantly higher than that of control neurons (Fig. 5.8), indicating that the level of NR1 surface expression in βSAP97-I2 expressing neurons is higher than control neurons.
Figure 5-7. Surface NMDAR currents in βSAP97-I2 expressing hippocampal neurons is significantly larger than that of control currents.

The amplitude of the surface NMDAR mediated current was measured in response to focal application of 1mM NMDA in either untransfected (control) or expressed βSAP97-I2-EGFP hippocampal neurons, * p<0.05.
Figure 5-8 Comparison of intensity of surface NR1 expression between control and βSAP97-I2.

Intensity of NR1 surface expression in βSAP97-I2 is significantly higher than that of control in immunocytochemistry, * p<0.05.
5.3. Discussion

In this study we have examined how βSAP97-I2 regulates AMPARs and NMDARs in hippocampal neurons. To date, very little is known about the effects of βSAP97-I2 on synaptic function.

5.3.1. βSAP97-I2 isoform regulates synaptic transmission and plasticity by sequestering AMPARs and NMDARs outside of synapses.

Our results indicate that βSAP97-I2 prevents LTP, decreases synaptic NMDARs and AMPARs and increases surface NMDARs and AMPARs which reveals that βSAP97-I2, like βSAP97-I3, localizes NMDARs and AMPARs to extrasynaptic sites. We also showed that in βSAP97-I2 expressing neurons, the small amplitude NMDA and AMPA EPSCs could not be increased with LTP, suggesting that they remain tightly bound /sequestered by βSAP97-I2 which cannot be released by LTP induction. We hypothesize that βSAP97-I2 tightly binds /sequesters NMDARs and AMPARs through its L-27 domain at extrasynaptic sites. Previous studies (Jeyifous et al., 2009) have reported that L27 domains are specific protein-protein interaction modules capable of forming heteromeric complexes which integrates multiple scaffold proteins into supramolecular assemblies; SAP97 shows a propensity for multimerization and homo-oligomerization via its N-terminal L27 domain (Howard et al., 2010). Therefore, through L-27 domain, SAP97 can regulate AMPARs at synapses (Rumbaugh et al., 2003) and NMDAR trafficking from ER to Golgi outposts (Jeyifous et al., 2009). As the L27 domain is common to both βSAP97-I2 and βSAP97-I3, we hypothesize that βSAP97-I2 may tightly bind /sequester NMDARs and AMPARs through its L-27 domain at extrasynaptic sites and this is likely to underlie this effect on pooling receptors at extrasynaptic sites. βSAP97 interacts with CASK in the hippocampus (Rumbaugh et al., 2003) and the βSAP97 interaction with CASK is essential for sorting NMDARs from ER to Golgi outposts (Rumbaugh et al., 2003; Waites et al., 2009), therefore we hypothesize that βSAP97-I2 may interact with CASK to stabilize NMDARs at extrasynaptic sites.

To date, we have only focussed on βSAP97-I2 overexpression experiments, however further experiments are required to determine whether synaptic and extrasynaptic AMPARs and NMDARs are rescued in the absence of βSAP97-I2. This requires the design of an shRNA to specifically target βSAP97-I2, enabling us to exclusively determine the role of this isoform in regulating the extrasynaptic pools of AMPA and NMDA receptors. We hypothesise that its role in receptor trafficking is to shift the dynamic equilibrium of glutamate receptors from a synaptic to an extrasynaptic location. Unfortunately, we have not been able to create the shRNA βSAP97-I2, because only a very short segment of the sequence is unique,
making it difficult to design a shRNA that will be specific for βSAP97-I2. Due to functional redundancy in loss-of-function experiments using SAP97 conditional KO mice, LTP was normal. This result indicates that other PSD-MAGUKs can presumably compensate for SAP97 in immature and mature neurons (Rumbaugh et al., 2003). Therefore the ideal condition of studying β- and αSAP97-I2 is in combination with deletions of other members of MAGUK family, for example, shRNA PSD95 as we previously employed for βSAP97-I3 (Chapter 3).

The reduction in synaptic AMPAR- and NMDAR-mediated EPSC amplitudes in βSAP97-I2-expressing neurons in our experiments may be a result of a decrease in AMPARs, NMDARs and/or synapse number. According to α-actinin puncta analysis data performed in parallel by J. Foote in our laboratory, overexpression of βSAP97-I2 significantly decreased the synapse numbers compared to controls (unpublished data). This result is consistent with a previous study (Rumbaugh et al., 2003) which demonstrated the loss of spines in βSAP97-I2 expressing neurons. Therefore this decrease in synapse numbers will directly contribute to the decreased synaptic AMPAR- and NMDAR EPSC amplitude in βSAP97-I2-expressing neurons that we observed. A previous report (Howard et al., 2010) showed the I3 insert but not the I2 insert binds to protein 4.1 and the actin/spectrin binding activity of protein 4.1 would be essential for the spine localization of SAP97-I3. In a subsequent report by Rumbaugh and colleagues (2003) (Howard et al., 2010; Li et al., 2011) it was indicated that the disruption of actin cytoskeleton would reduce the spine targeting efficiency of SAP97 in neurons. Furthermore, no loss of synapse number was reported in βSAP97-I3 expressing neurons (Howard et al., 2010). Therefore, the lack of binding of βSAP97-I2 to 4.1 protein could result in the loss of actin/spectrin binding activity of protein 4.1 and thus synapse number decreases in the dendrites. We also observed that the surface AMPAR- and NMDAR EPSC amplitudes were significantly higher than control. How can we explain this phenomenon occurring parallel to decreased synapse number in these neurons? One explanation is that there are large numbers of NMDARs and AMPARs expressed on the dendritic surface to compensate for the loss of synapse numbers, results in higher surface NMDAR- and AMPAR currents observed. However these receptors are not synaptic. Previous studies have shown that the I2 construct failed to increase the size of spines in neurons and the size of spines can correlate to the number of glutamate receptors (Hardingham & Bading, 2010; Milnerwood et al., 2010). Therefore, we hypothesize that large numbers of AMPARs and NMDARs are generated in the soma or local dendrites in βSAP97-I2 expressing neurons and exocytosis of AMPARs and NMDARs occurs along the dendrites where they are anchored by βSAP97-I2.

Although βSAP97 is major dominant in brain, it would be important to investigate the role of αSAP97-I2 in future investigations. RT-PCR results have confirmed the existence of
I2 and I3 splice variants of SAP97 in cortical culture and in the cerebellum (Hardingham & Bading, 2010). Combining our imaging and electrophysiology data (Chapter 3-5), we speculate the I3 insertion is dominant, because βSAP97-I3 rescues the deficits in AMPAR currents normally seen in PSD-93/-95 double-knockout neurons in hippocampal slice and in vivo (Howard et al., 2010).

Our data does not exclude an effect of βSAP97 I2 causing a decrease in glutamate release to cause the observed decrease in AMPAR and NMDAR EPSCs. However, presynaptic changes were not observed in immature neurons acutely expressing βSAP97 (Howard et al., 2010), suggesting the changes we observed in AMPA and NMDA EPSCs is not a result of decreased glutamate release. Thus, our results likely reflect that N-terminal βSAP97 isoforms regulate receptor targeting but not presynaptic function in early development.

Interestingly, in this study, we observed that NMDAR-mediated cLTP was clearly induced in control neurons (Fig.5.7), however, a number of previous studies have shown no LTP occurs in NMDAR-mediated currents, just in AMPAR-mediated currents (e.g. Nakagawa et al., 2004; Schluter et al., 2006; Howard et al., 2010). Contrary to what was expected, NMDAR-mediated cLTP was induced in our study, we hypothesize that this is due to the following reasons: (1) we used hippocampal culture rather acute slice or organotypic slice culture in our experiments; thus NMDARs and their binding partners may be able to move freely and rapidly in culture cells; (2) we used chemical LTP inducing protocol rather than electrical protocols. Chemical LTP protocol can activate neurons and the glycine can enhance NMDAR activity specifically, resulting in increasing functional NMDAR number at synapses; (3) we used paired whole cell recording rather than single cell recording plus extracellular field stimulation, and the paired recordings warrant only a few synapses to be stimulated at the same time in the whole experiments. There are few reports about cLTP and cLTD of NMDAR EPSCs, and their mechanisms are poorly understood. Therefore, NMDAR cLTP and cLTD may be a novel field in NMDAR functional studies, and hence our study may open a window for future studies in NMDAR plasticity. In addition, because NMDAR cLTP could be induced, this raises the possibility that increases in presynaptic glutamate release may occur during cLTP induction. In the present study, NMDAR cLTP could not be induced at βSAP97-I2 overexpressing neurons. As NMDARs were moved from synapses to extrasynaptic sites at βSAP97-I2 overexpressing neurons, it is not possible to increase NMDAR currents in these cells. Therefore, to clarify the locus of NMDAR-mediated enhancement, measurement of the paired pulse ratio (PPR) should be performed (Schluter et al., 2006).

Synaptic NMDAR and extrasynaptic NMDAR activities initiate different physiological and pathological signalling pathways (El-Husseini et al., 2000; Schluter et al., 2006),
However, the physiological function of extrasynaptic NMDARs is not fully understood (Zhou et al., 2008), therefore future studies are necessary to compare the synaptic NMDAR pool and NMDAR extrasynaptic pool during LTP and LTD during synaptic plasticity. Since we have previously shown that a significant proportion of AMPARs in βSAP97-I3 expressing neurons are located in the extrasynaptic membrane, we should examine whether the size of the surface pool of receptors is altered by the induction of synaptic plasticity in neurons expressing βSAP97-I2 and αSAP97-I2. Any changes observed in the size of the surface AMPAR currents could then be compared to the changes previously observed in synaptic AMPAR currents, providing insights into the redistribution of synaptic and extrasynaptic AMPAR pools during synaptic plasticity (Li et al., 2011).

Another future potential research direction for SAP97-I2 is the use of live cell imaging to examine its effects in AMPAR and NMDAR trafficking from ER to extrasynaptic sites. Our current data showed that βSAP97-I2 affects the distribution of NMDARs and AMPARs between synapse and extrasynaptic sites, so it will be interesting to investigate whether βSAP97-I2 co-trafficks with NMDAR or AMPAR from ER to extrasynaptic sites. One more avenue for future research is to understand the relationship between SAP97-I2, NMDARs and neurodegenerative diseases. Like βSAP97-I3, the βSAP97-I2 induced increases in extrasynaptic receptors, which may play a role in the altered NMDAR signalling and trafficking observed in conditions such as Alzheimer’s disease, Huntington’s disease, schizophrenia and addiction (Howard et al., 2010). In addition to studying this protein in vitro, further information on βSAP97I2 is required in vivo. Although, in vivo, SAP97 overexpression throughout synaptogenesis enhanced AMPAR and NMDAR currents and sped up NMDAR kinetics in mature neurons (Milnerwood et al., 2010), we do not know whether different isoforms of SAP97, including I2 and I3 isoforms, affect AMPAR and NMDAR currents in mature neurons in vivo. SAP97 is involved in glutamate receptors trafficking in vitro and vivo in early development (Milnerwood et al., 2010). However, a functionally distinct role for different insertions of SAP97 isoforms remains elusive.

5.4. Conclusions

We have performed functional and imaging studies that revealed that the β splice variant of the SAP97 I2 isoform regulated the synaptic localization of AMPARs and NMDARs, resulting in NMDARs and AMPARs recruitment to extrasynaptic sites in hippocampal neurons. Our present data showed that the βSAP97- I2 isoform is effective at sequestering both NMDARs and AMPARs away from synapses. Similar to βSAP97- I3, βSAP97-I2 might be a negative regulator of synaptic potentiation, favoring a shift towards
synaptic depression by sequestering both AMPARs and NMDARs at extrasynaptic sites. Our data reveal that the L-27 insert of βSAP97 isoforms likely underlies the ability of βSAP97 isoforms to sequester receptors at extrasynaptic receptors as this sequence is common to both βSAP97-I2 and βSAP97-I3. However, the I2 insert clearly regulates synapse density as the effect on synapse number was unique to I2 & not evident in I3-expressing neurons. Together these data show that βSAP97 isoforms exhibit both common and unique roles in regulating synapse structure and function in the brain.
Chapter 6. General Discussion

In this study, we have investigated how the SAP97 isoforms, αSAP97-I3, βSAP97-I3 and βSAP97-I2 affect glutamate receptors in hippocampal neurons. Overall, these SAP97 isoforms appear to have the important functional roles in regulating AMPA- and NMDA receptors: αSAP97-I3 and βSAP97-I3 demonstrate the strong effects on the expression levels and localisation of AMPARs and NMDARs at the PSD and extrasynaptic membrane. βSAP97-I2 also was revealed to have a potential role in regulating glutamate receptors at extrasynaptic membranes. These results, summarized in Table 6-1, demonstrate novel roles of SAP97 in controlling synaptic transmission and function. Together, the data presented here in this thesis further our understanding of the connection between MAGUK family and synaptic function and plasticity in the hippocampus.

6.1. αSAP97-I3 is an important positive regulator in clustering AMPARs at synapses.

As a recently identified isoform of SAP97 (Schluter et al., 2006), αSAP97-I3 and its function is only beginning to be defined at synapses. Due to the replacement of the prototypic N-terminal L27 domain with a putative palmitoylation motif, αSAP97-I3 functions differently from its sister isoform βSAP97-I3. These effects are also seen in another MAGUK family member, PSD95. Similar to SAP97, PSD95 exists in α- and β isoforms. This unique N-terminal palmitoylated α-isoform of PSD95 is predominant and directs targeting and clustering of AMPARs at synapses (El-Husseini et al., 2000; Schluter et al., 2006). Therefore functional redundancy and compensative mechanisms might exist between αSAP97 and αPSD95. The present results support the opinion that there is functional redundancy as our data have shown that overexpression of αSAP97-I3 rescued and restored miniature EPSCs in neurons in which PSD95 has been knocked down. In this study, in αSAP97-I3 expressing neurons NMDA receptors are recruited into the PSD the same as control neurons, suggesting αSAP97-I3 does not drive NMDARs into the synapse when over-expressed. However, we still don’t know if αSAP97-I3 affects NMDA receptor trafficking in other ways. Previous reports showed that CaMKII phosphorylates SAP97 on two major sites, one located in the N-terminal domain (Ser39) and the other in the first PDZ domain (Ser232), and phosphorylation of SAP97 control both NMDAR trafficking and insertion at synapses (Mauceri et al., 2007). However, we are lacking knowledge of αSAP97-I3 phosphorylation in NMDAR trafficking and insertion at synapses. It is likely that
Table 6-1 The effects of α-, βSAP97-I3 and βSAP97-I2 on AMPARs and NMDARs at synapses and extrasynaptic membranes

<table>
<thead>
<tr>
<th>SAP97 isoform</th>
<th>Role with AMPARs at synapses</th>
<th>Role with AMPARs at extrasynaptic membranes</th>
<th>Role with NMDARs at synapses</th>
<th>Role with NMDARs at the extrasynapse</th>
</tr>
</thead>
<tbody>
<tr>
<td>αSAP97-I3</td>
<td>Recruit AMPARs into the PSD and enhance EPSC amplitude</td>
<td>Excludes AMPARs from the extrasynaptic membrane</td>
<td>No apparent role in altering NMDARs at synapses</td>
<td>No apparent role in altering NMDARs at the extrasynaptic membrane</td>
</tr>
<tr>
<td>βSAP97-I3</td>
<td>Remove AMPARs from the PSD and decrease the amplitude of EPSCs</td>
<td>Recruits AMPARs to the extrasynaptic membrane</td>
<td>Excludes NMDARs from the PSD</td>
<td>Recruits NMDARs to the extrasynaptic membrane</td>
</tr>
<tr>
<td>βSAP97-I2</td>
<td>Remove AMPARs from the PSD and decrease the amplitude of EPSCs</td>
<td>Recruits AMPARs to the extrasynaptic membrane</td>
<td>Excludes NMDARs from the PSD</td>
<td>Recruits NMDARs to the extrasynaptic membrane</td>
</tr>
</tbody>
</table>

there was not CaMKII-dependent αSAP97-I3 phosphorylation on NMDARs in our experiments, as this potential phosphorylation would be involved in recruitment and insertion of NMDARs in the PSD and an enhanced NMDAR EPSCs which we did not observe.

Because αSAP97 is not the predominant SAP97 isoform in the brain, the essential roles of αSAP97-I3 underlying synapse physiology in brain have not been widely investigated. In this study, our data contribute and increase the knowledge of αSAP97-I3 in regulating glutamate receptors at synapses. However, the expression level and essential function roles of αSAP97-I3 under pathological conditions need to be investigated. It is possible that the expression level of αSAP97-I3 could be changed in pathological conditions such as neurodegenerative diseases. As neurons expressing αSAP97-I3 were very healthy
in our experiments, we hypothesize that the down-regulation of αSAP97-I3 level might happen in certain pathological conditions, but this remains to be tested. In addition to our electrophysiological data, for example enhanced mEPSCs frequency, increased baseline amplitude of EPSCs and occlusion of LTP, direct evidence to support αSAP97-I3 in stabilising glutamate receptors at synapses came from our Stanford colleagues’ data. Their data showed that αSAP97-I3 was located in the PSD through immuno-gold staining under electron microscopy. In other words, the unique N-terminal palmitoylation targets αSAP97-I3 to the PSD and creates docking sites for AMPARs and NMDARs within postsynaptic compartments. Our electrophysiological data further supported this opinion. In this study, we have observed the hugely enhanced amplitude of EPSCs in postsynaptic neuron expressing αSAP97-I3. Although this phenomenon was connected with the recruitment of AMPARs in the PSD, our data raise the possibility that αSAP97-I3 might direct and enable a healthy neurodevelopment, e.g. dendrite growth, stem cells migration and new born neuron integration. Indeed, a recent report showed that GluR1 controls dendrite growth through its binding partner, SAP97 (Zhou et al., 2008). In other words, SAP97 combining with GluR1 can control dendrite growth. However, this report did not further reveal which SAP97 isoforms control dendrite growth. According to our results, we propose that αSAP97-I3 is a positive regulator because it targets and clusters AMPARs in the PSD. We hypothesise that a positive regulator, such as αSAP97-I3, but not a negative regulator such as βSAP97-I3, will improve and maintain healthy neurodevelopment, because NMDAR-mediated synaptic currents lead to the build-up of a neuroprotective “shield” and promote neuronal health (Hardingham & Bading, 2010). We hypothesize that αSAP97-I3 through its binding partner, GluR1, regulates and controls dendrite growth and further increased the volume of the spine or the number of spines resulting in enhanced learning and memory in the brain. Because αSAP97-I3 is seen as a positive regulator of synapse function, it might aid hippocampal neurogenesis by improving new born cell growth. Because hippocampal adult neurogenesis is vital and essential for learning and memory (Neves et al., 2008), it will be of significant interest to investigate whether αSAP97-I3 is involved in recruiting AMPA or NMDA receptors and enhancing the integration of newborn neurons into existing neuronal circuits in the hippocampus.

In addition to in vitro work, to date the knowledge of αSAP97-I3 is lacking in vivo. A recent study showed that, in vivo, βSAP97 overexpression throughout synaptogenesis enhanced both AMPAR and NMDAR currents (Howard et al 2010). According to our current data in vitro, we hypothesize that αSAP97-I3 might have a functional role in affecting both AMPAR and NMDAR currents in vivo. Thus, it is interesting to clarify the roles of αSAP97-I3 in vivo in future work.
Comparison of the expression level of αSAP97-I3 and βSAP97-I3 under different conditions is of interest also. We hypothesize that the expression level of αSAP97-I3 and βSAP97-I3 might be changed underlying certain physiological and pathological conditions. Indeed, a recent report showed that SAP97 is developmentally upregulated and is required for synapse morphology and function (Mendoza-Topaz et al., 2008). However, to date, there are few reports to show the comparison of the expression level of αSAP97-I3 and βSAP97-I3 under physiological and pathological conditions. We presume that disrupting the balance between αSAP97-I3 and βSAP97-I3 might result in the alterations of gross expression levels of AMPAR- and NMDA receptors at synapses, and these alternations may be involved in the pathological pathways.

6.2. βSAP97-I3 and βSAP97-I2 are important regulators in clustering AMPARs and NMDARs at extrasynaptic sites

βSAP97, the predominant isoform of SAP97, is expressed at presynaptic and postsynaptic sites and perisynaptically in dendritic spines. Because germline KO causes lethal feeding deficits in neonatal mice, this has made it difficult to ascertain SAP97’s synaptic function (Muller et al., 1995; Valtchanoff et al., 2000; Caruana & Bernstein, 2001; Waites et al., 2009; Howard et al., 2010). Thus, in this study, βSAP97-I3 and βSAP97-I2 isoforms were specifically studied in hippocampal neurons in vitro. Our data showed for the first time that overexpression of βSAP97 isoforms removed both NMDARs and AMPARs from synaptic sites and recruited them into extrasynaptic sites (Table 6-1). Because NMDARs are vital for LTP and LTD, it will be important to investigate NMDAR trafficking between extrasynaptic and synaptic pools in βSAP97 expressing neurons. A number of studies suggest that the consequences of NMDAR activation can vary widely depending on whether synaptic or extrasynaptic receptors are activated (Lozovaya et al., 2004; Thomas et al., 2005; Harris & Pettit, 2007; Zhang et al., 2007). Recent studies demonstrate that synaptic NMDAR transmission drives neuroprotective gene transcription, whereas extrasynaptic NMDAR activation promotes cell death. A recent paper (Milnerwood et al., 2010) showed that increases in extrasynaptic NMDAR signaling and expression contributes to the phenotype onset in Huntington’s Disease mice (Liu et al., 2004; Massey et al., 2004). In this paper (Milnerwood et al., 2010), investigators showed specific increases in extrasynaptic NMDAR expression, current, and associated reductions in nuclear cAMP response element-binding (CREB) activation in Huntington’s Disease mouse striatum; moreover, pharmacological block of extrasynaptic NMDARs with memantine reversed signaling and motor learning deficits (Milnerwood et al., 2010). Our βSAP97 isoform data in
present study raise the possibility that βSAP97 might be involved in the pathological pathway in Huntington’s Disease as well. The use of NMDA receptor antagonists as treatments to control extrasynaptic NMDAR currents for Huntington’s Disease is not a viable option. The reason is that although NMDARs are an attractive candidate target for therapeutic intervention in Huntington’s Disease, these receptors are critical to many processes and broad intervention of NMDAR activity produces serious side effects (Chen & Lipton, 2006; Milnerwood et al., 2010). Therefore, we hypothesize that downregulating the expression level of βSAP97 isoforms to control extrasynaptic NMDAR signalling in Huntington’s Disease might be a potential new therapeutic target in the future. Consistent with extrasynaptic NMDAR signalling contributing to Huntington’s Disease, we have observed that neurons expressing βSAP97-I3 and βSAP97-I2 were more difficult to record with whole cell patch clamp. This suggests that the βSAP97 isoforms were tipping the balance towards cell death by increasing extrasynaptic NMDA receptors.

In addition to Huntington’s disease, SAP97’s role in brain diseases has been raised in schizophrenia, Alzheimer’s disease, Parkinson’s disease and epilepsy (Wakabayashi et al., 1999; Nash et al., 2005; Finardi et al., 2006; Sato et al., 2008; Fukataa et al., 2010; Hammond et al., 2010; Marcello et al., 2010). One study (Marcello et al., 2007) showed SAP97 as a key element of molecular pathogenesis in Alzheimer’s disease, demonstrating that the ADAM10/SAP97 complex is markedly reduced and that GluR1/SAP97 interaction is significantly increased in hippocampus of Alzheimer’s disease patients. This indicates that alterations in SAP97 are a key element of molecular pathogenesis of the disease (Macklin, 2010). Another report revealed that an increase in the expression level of SAP97 occurs in the dorsolateral prefrontal cortex in schizophrenia patients (Sato et al., 2008). SAP97 is also implicated in Parkinson’s disease, where total membrane and synaptic SAP97 levels were dramatically reduced in the dopamine-depleted striatum, indicating abnormalities in SAP97 level may contribute to processes that are responsible for parkinsonism (Nash et al., 2005). In cerebral heterotopia, NR2A and NR2B downregulation was accompanied by a reduction of SAP97, thus suggesting that NMDAR impairment was associated with altered molecular structure of the postsynaptic membrane and may contribute to the genesis of epileptic phenomena (Finardi et al., 2006). We hypothesize that βSAP97 isoforms that enhance extrasynaptic NMDAR currents directly or at least in part, contributing to these neurodegenerative diseases.

βSAP97 isoforms can regulate NMDAR currents, but it is unknown whether βSAP97 isoforms can alter the subunit composition of the NMDARs during synaptic plasticity. Calcium influx depends on NR2B expression, as NR2B confers a slower decay time to the receptor and excessive excitatory neurotransmitter release activates extrasynaptic receptors, which causes neurotoxicity (Ivanov et al., 2006; Leveille et al., 2008). Pioneer
studies showed that SAP97 is directly associated with NR1/NR2A receptors through its PDZ1 domain and with the NR2B C-terminal tail (Gardoni et al., 2003; Wang et al., 2005; Cousins et al., 2007). Previous studies indicated that the subunit composition of synaptic and extrasynaptic NMDA receptor pools is similar at early developmental stages, with NR2B subunits present in both compartments but then the NR2B-dependence of LTP disappears in older slices (Harris & Pettit, 2007; Foster et al., 2010). However, there are controversies about specific NMDAR subunit involvement in synaptic plasticity (Foster et al., 2010). For example, some studies showed that activation of NR2A-NMDARs is specifically important for LTP induction, whereas NR2B-NMDARs are specifically involved in LTD induction (Liu et al., 2004; Massey et al., 2004). However, other work showed that the NR2B subunit plays a critical role in LTP and by contrast, NR2A is not essential for LTP (Foster et al., 2010). Therefore, it is important to clarify the relationship between βSAP97 isoforms and the subunit composition of the NMDARs involved in our future experiments. In these experiments, it may be necessary to use NR2B NMDAR subunit selective antagonist ifenprodil (Williams, 2001) and NR1, NR2A and NR2B immunoreactivity to determine subcellular localization for NMDAR subunits at βSAP97 isoform expressing neurons in synaptic plasticity.

In addition, due to function redundancy between MAGUK family members, βSAP97-I3 down-regulation of synaptic functions might be compensated by other members of MAGUK family like αPSD95. We hypothesize that under normal physiological conditions, this balance is working very well. For instance, while βSAP97-I3 recruits NMDARs & AMPARs to extrasynaptic sites, αPSD95 could coordinate AMPAR and NMDAR exocytosis and insert them into the PSD, making the balance of extrasynaptic and synaptic pools correct. Any disruption to this balance would then activate a pathological pathway.

In addition to NMDARs, βSAP97-I3 & I2 affected extrasynaptic and synaptic AMPAR pools as well. Previous work showed that during scaffold protein assembly via L27 domains, other protein-binding domains such as the PDZ and SH3 domains would be free to recruit their specific target proteins receptors, ion channels and their downstream partners. Therefore L27 domain–containing scaffold proteins are ideally suited for assembly of supramolecular signaling complexes (Feng et al., 2004). Because βSAP97-I3 and βSAP97-I2 contain an L-27domain and SH3 and GK domains, these functional domains might endow βSAP97-I3 and βSAP97-I2 the ability to assembly of supramolecular complexes.

In addition to neurons, SAP97 regulates the function of glia cells in brain. SAP97 acts to stop active myelination in Schwann cells (Macklin, 2010). Myelination is vital to maintain fast neuronal transmission in the peripheral nervous system (PNS) and in the central nervous system (CNS). However, we still don't know which SAP97 isoforms are involved in
controlling myelination development. It will also be important to determine other roles that SAP97 may play in glia.

In addition to the brain, SAP97 isoforms are expressed widely in other tissues. For example, SAP97 is the main MAGUK protein expressed in cardiomyocytes (Godreau et al., 2002; Petitprez et al., 2011). SAP97 regulates the targeting and localization of cardiac potassium and sodium channels, such as Kv4.2, Kv4.3, Kir2.x11, Kv1.5 and Nav1.5 channels in cardiomyocytes (Nattel et al., 1999; Alexander et al., 2009; Petitprez et al., 2011; Ting et al., 2011). However, these studies have not investigated individual SAP97 isoforms. Thus, it would be interesting to test the functional roles of βSAP97-I3 and βSAP97-I2 in cardiomyocytes, particular in affecting Ca^{2+} channels, because alternations in Ca^{2+} current is seen in heart failure (Bers et al., 2003). To our knowledge, there are not any reports for Ca^{2+} channel regulation by SAP97. Due to βSAP97 playing a role as a negative regulator of glutamate receptors in hippocampal neurons, we hypothesise that βSAP97-I3 and βSAP97-I2 might affect Ca^{2+} current in cardiomyocytes. In addition to cardiomyocytes, SAP97 plays important roles in regulating smooth muscle orientation in the mouse ureter, and SAP97 knockout mice develop severe urinary tract abnormalities, including congenital hydronephrosis, which is the leading cause of renal failure in infants and children (Zhen et al., 2006). We hypothesize that βSAP97-I3, βSAP97-I2 and αSAP97-I3 might have a functional role in regulating smooth muscle in the ureter. Moreover, SAP97 is targeted by cancer-related human papillomavirus (HPV) and this raises the possibilities that SAP97 might be involved in different type’s of cancers including glial tumor e.g. astrocytomias, glioblastoma multiforme in the brain. However, to date, little is known about βSAP97-I3 and βSAP97-I2 involvement in these tumours. Although the obstacle of studying SAP97 isoforms is that there is complicated functional redundancy between the members of MAGUK and member isoforms in MAGUK family, overexpression, knockdown and knockout of each isoform are effective approaches to study SAP97 roles.

In summary, in this study, our data reveal that there are functional distinctions between βSAP97-I3, βSAP97-I2 and αSAP97-I3. Particularly, these SAP97 isoforms target AMPARs and NMDARs trafficking at synaptic and extrasynaptic sites during synaptic plasticity in hippocampal neurons. Whether these isoforms work in tandem or alone remains to be determined, however, our data indicate that SAP97 isoforms could play important functional roles in brain regions in which this protein is enriched underlying both physiological and pathological conditions.
References


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