Modulation of AMPA receptor trafficking and its involvement in synaptic plasticity and spatial memory

by

Yuan Ge

B.Sc., Peking University, 2002

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate Studies (Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA (Vancouver)

April, 2010

© Yuan Ge, 2010
ABSTRACT

The α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) subtype of glutamate receptors mediates the fast excitatory synaptic transmission in the mammalian brain. Increasing evidence suggests that rapid movement of AMPARs into and out of synapses is the major mechanism for changes in synaptic strength, which is thought to underlie learning and memory. Although the bidirectional hippocampal synaptic plasticity, particularly long-term potentiation (LTP) and long-term depression (LTD), has been regarded as a candidate mechanism for long-term spatial memory (LSM), the precise contribution of LTP and LTD to LSM remains poorly understood. Using antagonists that target NMDARs carrying specific NR2 subunits, we found that LTP and LTD in freely moving rats can be selectively abolished by NR2A and NR2B antagonists, respectively. Using the Morris water maze, we found that only the NR2B antagonist disturbed the consolidation of LSM. In addition, a similar LSM deficit was observed when the expression of LTD was prevented by inhibiting regulated AMPAR endocytosis. Thus, these findings support a functional requirement of hippocampal LTD in the consolidation of LSM.

Blocking LTP by NR2A-preferential antagonist had no effects on LSM. However, another structurally and mechanistically different LTP-specific inhibitor is still lacking. Since the expression of LTP is thought to be mediated by the facilitated exocytosis of AMPARs, we therefore attempted to identify novel AMPAR binding partner(s) using co-immunoprecipitation with anti-GluR1 or anti-GluR2 antibody followed by mass spectrometric analysis. We found that p97, also called valosin-containing protein (VCP), specifically interacts with and modulates trafficking of homomeric GluR1 receptors.
Using various truncated and deleted constructs of GluR1, we found that p97 interacts with N-terminal of GluR1, but not GluR2, resulting in facilitated formation of homomeric GluR1 receptors by decreasing GluR1/GluR2 heteromeric receptors formation. Moreover, we found that under basal conditions, p97 retained homomeric GluR1 AMPARs in the intracellular pool, but immediately after the induction of LTP, it disassociated from GluR1 and hence, allowed these homomeric AMPARs insert into the postsynaptic membrane, thereby contributing to LTP expression. Thus, our results highlight a previously unknown molecular mechanism by which p97 regulates formation and trafficking of homomeric GluR1 AMPARs, and thereby plays a critical role in LTP expression.
# TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................... ii
TABLE OF CONTENTS ........................................................................................................................ iv
LIST OF TABLES ................................................................................................................................... viii
LIST OF FIGURES ............................................................................................................................. ix
LIST OF ABBREVIATIONS ..................................................................................................................... x
ACKNOWLEDGEMENTS ....................................................................................................................... xii
CO-AUTHORSHIP STATEMENT ......................................................................................................... xiii
1 INTRODUCTION ............................................................................................................................. 1
  1.1 Glutamate receptors ................................................................................................................... 1
    1.1.1 AMPA receptors ................................................................................................................. 2
    1.1.2 NMDA receptors ................................................................................................................. 5
  1.2 AMPAR trafficking and synaptic plasticity .................................................................................. 7
    1.2.1 Vesicular trafficking, exocytosis, endocytosis and lateral diffusion of AMPARs .................. 8
    1.2.2 AMPAR phosphorylation .................................................................................................... 10
    1.2.3 AMPAR interacting proteins ............................................................................................. 14
    1.2.4 Ca\(^{2+}\)-permeable AMPAR and synaptic plasticity .......................................................... 18
  1.3 NMDAR and synaptic plasticity .................................................................................................. 21
    1.3.1 Characteristics of NR2A and NR2B subunits ..................................................................... 21
    1.3.2 Interacting proteins of NR2A and NR2B subunits ............................................................... 23
    1.3.3 Roles of NR2A and NR2B in LTP and LTD ......................................................................... 25
  1.4 Synaptic plasticity and spatial memory ....................................................................................... 28
    1.4.1 Synaptic plasticity and memory (SPM) hypothesis ............................................................. 28
    1.4.2 Hippocampus and spatial memory ...................................................................................... 29
    1.4.3 Hippocampal LTP and spatial memory .............................................................................. 31
    1.4.4 Hippocampal LTD and spatial memory .............................................................................. 35
  1.5 p97/VCP ...................................................................................................................................... 38
    1.5.1 p97 structure ...................................................................................................................... 39
    1.5.2 Diverse biological functions of p97 .................................................................................... 40
    1.5.3 Modulation of p97 activities ............................................................................................... 44
    1.5.4 p97 and NSF ....................................................................................................................... 46
3.2.5 GST pull down assay ................................................................. 125
3.2.6 Biotinylation ............................................................................ 126
3.2.7 Immunofluorescent microscopy.................................................. 126
3.2.8 Electrophysiological recording in HEK 293 cells ....................... 127
3.2.9 Hippocampal neuron culture, LTP and LTD induction, and transfection ..... 128
3.2.10 Recordings of miniature excitatory postsynaptic currents in cultured hippocampal neurons .............................................................. 128
3.2.11 Statistical analysis .................................................................... 130
3.3 Results .......................................................................................... 131
3.3.1 Mass spectrometric analysis of novel AMPAR associated proteins .......... 131
3.3.2 p97 interacts with homomeric GluR1 AMPARs .............................. 138
3.3.3 p97 interacts with multiple domains within N-terminal of GluR1 ......... 139
3.3.4 Changes in surface expression level and channel gating properties of homomeric GluR1 AMPARs by p97 ........................................... 145
3.3.5 p97 facilitates the formation of homomeric GluR1 AMPARs and keeps them in intracellular sites .............................................................. 151
3.3.6 p97 keeps homomeric GluR1 AMPARs in the intracellular pool in cultured hippocampal neurons .............................................................. 158
3.3.7 LTP, but not LTD induces dissociation of p97-GluR1 interaction ..... 159
3.3.8 Insertion of homomeric GluR1 AMPARS into postsynapses shortly after glycine stimulation in cultured hippocampal neurons ......................... 165
3.3.9 Overexpression of p97 abolished LTP induced by glycine in cultured hippocampal neurons ...................................................................................... 168
3.4 Discussion ...................................................................................... 172
3.4.1 Difference between p97 and NSF ............................................... 173
3.4.2 p97 is a novel AMPAR N-terminal associated protein .................. 173
3.4.3 p97 facilitates homomeric GluR1 AMPAR formation .................... 175
3.4.4 p97 modulates homomeric GluR1 AMPAR trafficking ................. 176
3.5 Supplementary materials ................................................................ 179
3.5.1 Methods for plasmid construction ............................................. 179
3.6 References .................................................................................... 182
4. CONCLUDING REMARKS .............................................................. 188
4.1 Research summary ........................................................................ 188
4.2 Discussion .................................................................................... 191
LIST OF TABLES

Table 3.1 Peptide summary of p97 (VCP) identified by mass spectrometric analysis... 134
LIST OF FIGURES

Figure 2.1 NR2A and NR2B antagonists prevent the formation of hippocampal LTP and LTD, respectively, in freely moving adult rats ................................................................. 87
Figure 2.2 LSM formation is affected if the induction of hippocampal LTD is prevented during MWM training ....................................................................................................... 91
Figure 2.3 Impact of pre-training injection of Ro on LSM formation was not caused by state-dependent learning ................................................................................................... 95
Figure 2.4 LSM formation is affected if the expression of hippocampal LTD is inhibited during MWM training ....................................................................................................... 97
Figure 2.5 Acquisition of spatial memory does not require hippocampal LTD .......... 103
Figure 2.6 Consolidation of spatial memory was abolished by Tat-GluR23Y peptide.... 105
Figure 2.7 Retrieval of spatial memory does not require hippocampal LTD .......... 107
Figure 2.8 Retrieval of spatial memory does not require LTP ................................. 108

Figure 3.1 Co-immunoprecipitation to identify novel AMPAR associated proteins..... 132
Figure 3.2 p97 selectively interacts with homomeric GluR1 AMPARs....................... 136
Figure 3.3 The interaction domain is within the N-terminal of GluR1 ....................... 140
Figure 3.4 Multiple domains within the N-terminal of GluR1 are involved in p97-GluR1 interaction ....................................................................................................................... 143
Figure 3.5 Interaction with p97 decreases cell surface expression of GluR1 ............ 146
Figure 3.6 p97 changes the channel gating properties of homomeric GluR1 AMPARs 150
Figure 3.7 p97 decreases the formation of GluR1/GluR2 heteromeric receptors in COS-7 cells ................................................................................................................................. 152
Figure 3.8 p97 depresses the cell surface expression of homomeric GluR1 receptors in the GluR1/GluR2 cotransfected HEK 293 cells .................................................................. 155
Figure 3.9 p97 inhibits the postsynaptic surface expression of homomeric GluR1 receptors in hippocampal neurons .................................................................................. 160
Figure 3.10 Dissociation of p97-GluR1 after LTP induction in cultured hippocampal neurons .......................................................................................................................... 163
Figure 3.11 Synaptic insertion of homomeric GluR1 receptors after LTP induction in cultured hippocampal neurons .................................................................................. 166
Figure 3.12 Overexpression of p97 abolished glycine induced LTP in cultured hippocampal neurons ................................................................................................. 170
LIST OF ABBREVIATIONS

AAA: ATPase associated with a variety of activities
ABP: AMPA-binding protein
AKAP79: A-kinase anchoring protein 79
AMPA: \( \alpha \)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
CaMKII: calcium/calmodulin-dependent protein kinase II
CNS: central nervous system
ERAD: ER-associated degradation
EPSC: excitatory postsynaptic current
fEPSP: field excitatory postsynaptic potentials
GRIP: glutamate receptor-interacting protein
HFS: high-frequency stimulation
LSM: long-term spatial memory
LFS: low-frequency stimulation
LTD: long-term depression
LTP: long-term potentiation
MAGUK: membrane-associated guanylate kinase
MTL: medial temporal lobe
MWM: Morris water maze
Narp: neuronal-activity-regulated pentraxin
NMDA: N-methyl-D-aspartate
NP1: neuronal pentraxin 1
Npl4: nuclear protein localization 4
NPR: neuronal pentraxin receptor
NSF: N-ethylmaleimide-sensitive fusion protein

PICK1: protein interacting with C-kinase 1

PKA: cAMP-dependent protein kinase

PKC: protein kinase C

PSD-95: postsynaptic density protein-95

RasGAP: Ras GTPase activating protein

Ras-GRF1: Ras-guanine nucleotide-releasing factor 1

SAP102: synapse-associated protein 102

SAP97: synapse-associated protein 97

SNAP: soluble NSF attachment protein

SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptors

SRF: serum response factor

TARP: AMPA receptor regulatory protein

t-SNARE: target-soluble NSF attachment protein receptor

Ufd1: ubiquitin fusion degradation 1

VCIP135: VCP(p97)/p47 complex-interacting protein, p135

VCP: valosin-containing protein

VDCC: voltage-dependent Ca²⁺ channel

ZIP: myristoylated zeta-pseudosubstrate inhibitory peptide
ACKNOWLEDGEMENTS

I would like to offer my gratitude to my supervisor Dr. Yu Tian Wang for his excellent guidance, support and encouragement of my work. I especially enjoyed working in the Dr. Wang’s lab from where I learned a great deal about experimental neuroscience. I am very grateful to Dr. Jie Lu, Dr. Zhifang Dong, Dr. Lidong Liu, Dr. Dongchuan Wu, Dr. John G. Howland and Mr. Taesup Cho in Dr. Yu Tian Wang’s lab, as well as Ms. Rosemary A. Bagot in Dr. Tak Pan Wong’s lab and Ms. Shujun Lin in Dr. Juergen Kast’s lab, for collaborating on some of the studies described in this thesis. I would also like to thank Dr. Yuping Li for her technical assistance and Ms. Agnes Kwok for the thesis editing.

I would like to thank my supervisory committee members Drs Lynn A. Raymond, Anthony G. Phillips and Brian A. MacVicar for their continuous support, helpful suggestions and critical review of my thesis.

Special thanks are owed to my parents, my husband Yuxin, and my son David. This work could never be done without their love and support.
CO-AUTHORSHIP STATEMENT

This work was designed by Dr. Yu Tian Wang and myself. The experiments, data analysis and the manuscript writing were mainly done by me, except work in Fig. 2.1 and Fig. 2.4A was done by Z. Dong (Dr. Yu Tian Wang’s lab); work in Fig. 2.4E, Fig. 2.5 and Fig. 2.6 was done by R.C. Bagot (Dr. Tak Pan Wong’s lab); plasmid construction in Fig. 3.3B and Fig 3.3C was done by J. Lu (Dr. Yu Tian Wang’s lab); experiments in Fig. 3.5D and Fig. 3.6 were done by D. Wu (Dr. Yu Tian Wang’s lab); experiments in Fig 3.9 and Fig 3.11 were done by L. Liu (Dr. Yu Tian Wang’s lab); and mass spectrometric analysis in Table 3.1 was done by S. Lin (Dr. Juergen Kast’s lab).
1 INTRODUCTION

Synaptic transmission is one of the fundamental processes that all brain functions are based on. Moreover, changes in the strength of neuronal connections are crucial for information processing which are encoded and stored in the central nervous system. Synaptic transmission can be regulated by changes in transmitter release or in the function of neurotransmitter receptors. The α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) subtype of glutamate receptors mediates the majority of fast excitatory synaptic transmission in the mammalian brain. In addition, rapid trafficking of AMPA receptors in and out of the postsynaptic membrane is proposed to be a major mechanism for synaptic plasticity. Thus, understanding the cellular mechanisms that modulate AMPAR trafficking will be crucial for understanding the cellular basis of learning and memory.

1.1 Glutamate receptors

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (von Kitzing et al., 1994). The glutamatergic system is important for synaptic plasticity (Asztely and Gustafsson, 1996; Bear and Abraham, 1996; Larkman and Jack, 1995; Voronin, 1994), which underlies many advanced brain function such as learning and memory. Also, excessive glutamate release or overstimulation of glutamate receptors are believed to be the main reason in causing neuronal death in certain pathological conditions, such as stroke and other forms of neurotrauma (Chen et al., 1995; Colwell and Levine, 1996; Doble, 1995; Fujisawa et al., 1993).
Glutamate receptors are divided into two groups: ionotropic and metabotropic receptors, differing in their molecular, pharmacological, and physiological properties (Hollmann and Heinemann, 1994; Kew and Kemp, 2005). The ionotropic glutamate receptors have been classified into three major subtypes, AMPA, kainate, and N-methyl-D-aspartate (NMDA) receptors, named after their most selective agonist (Watkins et al., 1981); the metabotropic glutamate receptors are G-protein coupled receptors mediating slow and prolonged responses (Doble, 1995; Swope et al., 1999). As AMPA and NMDA receptors are the main mediators for fast synaptic transmission, only these two types of glutamate receptors will be discussed in more details.

1.1.1 AMPA receptors

AMPA receptors (AMPARs) are responsible for the primary depolarization in glutamate-mediated neurotransmission and play key roles in synaptic plasticity. AMPARs consist of four closely related genes, with about 70% sequence homology (Collingridge et al., 2004), that encode the four subunits GluR1-4 or A–D (Laube et al., 1998; Mano and Teichberg, 1998; Rosenmund et al., 1998). Each subunit contains a large extracellular amino (N)-terminal domain, three trans-membrane spanning domains (TM1, TM3 and TM4), a re-entry hairpin loop (M2) that forms the pore-lining region, an extracellular loop (S2) between TM3 and TM4, and an intracellular carboxy (C)-terminal domain. The N-terminal segment is homologous to the bacterial leucine-isoleucine-valine binding protein (LIVBP), while the adjacent ligand-binding domain (LBD) is homologous to glutamine binding protein (Madden, 2002). The LBD is split into the S1 and S2 segments by TM segments. Ligand binding to the LBD initiates conformational changes that are transduced to the TM segments and trigger opening of the channel’s gate.
The intracellular C-terminals are distinct from each other. GluR1, GluR4 and an alternative splice form of GluR2 (GluR2L) have long C-terminals, while the predominant splice form of GluR2, GluR3 and an alternative splice form of GluR4 (GluR4S) have short C-terminals. The distinction of C-terminals determines the binding of the subunits to the specific interacting proteins as well as phosphorylation by different protein kinases (Song and Huganir, 2002).

The four AMPAR subunits, GluR1-4, assemble in different combination to form tetrameric channels (Rosenmund et al., 1998). AMPARs are widely expressed in the brain with different distribution for subunits composition. GluR1, GluR2 and GluR3 subunits are enriched in the outer layers of the cerebral cortex, hippocampus, olfactory regions, basal ganglia, lateral septum and amygdala (Beneyto and Meador-Woodruff, 2004; Keinanen et al., 1990). The GluR4 subunit is present in lower amounts throughout the central nervous system (CNS), except in the reticular thalamic nuclei and the cerebellum, where this subunit is also abundant (Martin et al., 1993; Petralia and Wenthold, 1992; Spreafico et al., 1994). In hippocampal pyramidal cells, the AMPA receptor subtypes that predominate are composed of GluR1/2- and GluR2/3-containing receptors (Wenthold et al., 1996). However, recent work (Ju et al., 2004; Kumar et al., 2002; Plant et al., 2006; Terashima et al., 2004) suggests that principal neurons contain substantial intracellular reserve pools of GluR2-lacking AMPARs (GluR1 homomers), which may be synaptically incorporated under specific conditions, such as long-term potentiation (LTP).

How do the AMPAR subunits form different subunit composition? AMPAR subunits are synthesized and assembled as dimers of dimers (Ayalon et al., 2005; Ayalon
and Stern-Bach, 2001; Tichelaar et al., 2004) in the rough endoplasmic reticulum (ER) and then inserted into the plasma membrane after crossing the Golgi apparatus. The signal sequence in the beginning of the N-terminal, which is recognized by the Sec61 channel, is co-translationally inserted into rough ER once synthesized, and the following biogenesis of AMPARs occurs at the ER membrane (Clemons et al., 2004). The precise mechanisms that govern subunit assembly, especially the different combinations, are not well understood. Dimerisation of AMPAR subunits have been shown to prefer to form heteromers rather than simply homodimers (Mansour et al., 2001). How this first assembly step is achieved is unclear, but it is mainly due to the tight interaction between N-terminals of different subunits (Kuusinen et al., 1999). Furthermore, tetramers form from dimers, which requires the extracellular S2 loop and the transmembrane segments (including the pore loop) (Ayalon and Stern-Bach, 2001).

The subunit composition of AMPARs determines channel gating kinetics and trafficking properties. Due to the RNA editing of the critical glutamine 607 residue (Q607) to arginine (R607) in the pore-lining (M2) region of GluR2, GluR2-containing AMPARs are Ca\(^{2+}\)-impermeable, show electrically linear or outward rectification in their current/voltage (I/V) relationships, and are resistant to blockade by polyamines; while GluR2-lacking AMPARs are Ca\(^{2+}\)-permeable, show electrically inward rectification, exhibit a high single-channel conductance, and are blocked in a voltage-dependent manner by endogenous polyamines. Although the majority of AMPARs in the CNS are GluR2-containing, significant expression of Ca\(^{2+}\)-permeable AMPARs is seen in neuronal and glial cells of various brain regions. It is now becoming apparent that the
regulation of these Ca\(^{2+}\)-permeable AMPARs is crucial in synaptic plasticity, neuronal
development and neurological diseases.

**1.1.2 NMDA receptors**

NMDA receptors are also heteromeric molecules, being formed of NR1, NR2, and NR3 subunits. To date, a single NR1, with eight splice variants, four NR2 subunits (NR2A-D), and two NR3 subunits (NR3A and NR3B) have been identified (Cull-Candy and Leszkiewicz, 2004). Similar to AMPAR subunits, each NMDAR subunit contains a large N-terminal extracellular domain, three membrane-spanning domains, a re-entry or ‘hairpin’ loop that forms the pore-lining region (membrane domain 2) and an intracellular C-terminal domain. However, NMDARs exhibit remarkable properties that distinguish them from AMPARs. Firstly, NMDARs show voltage-dependent block by physiological levels of extracellular Mg\(^{2+}\), and are highly permeable to Ca\(^{2+}\), which is because of a critical asparagine residue located within the re-entrant pore loop (Cull-Candy and Leszkiewicz, 2004). Since the release of Mg\(^{2+}\) blockade can be achieved at depolarized membrane potentials, this property makes the NMDAR a molecular coincidence detector, permitting activation of the receptor only when pre- and postsynaptic cells are excited synchronously. Moreover, the initial activation of NMDAR requires the presence of not only glutamate, but also the coagonist glycine (Thomson, 1990). Furthermore, compared with AMPARs, the excitatory postsynaptic currents that result from NMDAR activation exhibit slow kinetics with a rise time of about 10 ms and a decay time of 100 ms (Cull-Candy and Leszkiewicz, 2004).

Functional NMDARs are heteromtetromers containing two glycine-binding NR1 subunits and two glutamate-binding NR2 subunits. The identity of the NR2 subunit
determines many of the biophysical and pharmacological properties of NMDARs (Cull-Candy and Leszkiewicz, 2004). The NR2A subunit confers a lower affinity for glutamate, faster kinetics, greater channel open probability, and more prominent Ca\textsuperscript{2+}-dependent desensitization followed by the NR2B subunit, which confers slower channel kinetics and reduced open probability. The NR2C and NR2D subunits are characterized by slowest channel kinetics, low conductance openings, and reduced sensitivity to Mg\textsuperscript{2+} blockade (Cull-Candy and Leszkiewicz, 2004). In addition, the composition of NMDARs varies in different brain regions. In adults, NR2A is ubiquitously expressed in the brain, NR2B is mostly restricted to the forebrain, NR2C is mostly restricted to the cerebellum, and NR2D is expressed in small number of cells in selected brain regions (Papadia and Hardingham, 2007). The most widely expressed NMDARs consist of the obligate subunit NR1 plus either NR2A or NR2B or a mixture of two (Waxman and Lynch, 2005). NR2B is expressed at high levels in early developmental stages and declines postnatally, whereas expression level of NR2A increases postnatally and maintains at high level throughout adulthood (Kohr, 2006). It has been proposed that NR2A-containing NMDARs are more likely to occupy the central portion of the synapse, while NR2B-containing NMDARs are preferentially targeted to peripheral portions of the synapse or to extrasynaptic sites (Dalby and Mody, 2003; Townsend et al., 2003; Zhao and Constantine-Paton, 2007).
1.2 AMPAR trafficking and synaptic plasticity

Glutamatergic synapses undergo activity-dependent long-lasting changes in synaptic strength, long-term potentiation (LTP) or long-term depression (LTD), which are thought to underlie learning and memory formation (Martin et al., 2000; Morris, 2006). LTP is characterized by a persistent increase in the efficacy of synaptic transmission, whereas LTD refers to a persistent decrease of synaptic transmission. LTP and LTD can be induced by a short period of high-frequency synaptic stimulation or a period of low-frequency synaptic stimulation, respectively (Morris, 2006). In addition, pharmacological stimulation of excitatory synapses also induces LTP or LTD, named chemical LTP or LTD (Broutman and Baudry, 2001; Lu et al., 2001b; Park et al., 2004). Increasing evidence suggests that rapid movement of AMPARs into and out of synapses is the major mechanism for changes of synaptic strength. Unlike NMDARs, AMPARs constitutively cycle between intracellular stores and the cell surface, and the cycling can be regulated by the changes of synaptic transmission (Bredt and Nicoll, 2003; Collingridge et al., 2004; Malinow and Malenka, 2002; Sheng and Kim, 2002; Song and Huganir, 2002). Moreover, lateral movement of AMPARs allows the exchange of the receptor location between the extrasynaptic and synaptic membranes (Choquet and Triller, 2003). Although the mechanisms that modulate AMPAR trafficking are still not clear, several lines of evidence have shown that AMPAR phosphorylation/dephosphorylation and interaction with other proteins control their recycling and localization to defined postsynaptic sites, thereby regulating the strength of synapses (Santos et al., 2009).
1.2.1 Vesicular trafficking, exocytosis, endocytosis and lateral diffusion of AMPARs

Fast changes in the number of synaptic AMPARs, which play an important role in synaptic plasticity, are probably achieved by a combination of direct insertion/removal of AMPARs at synaptic and/or extrasynaptic sites and by their lateral diffusion to and from synapses. AMPARs traffic between the plasma membrane and the intracellular compartments via vesicle mediated membrane fusion (exocytosis) and internalization (endocytosis). AMPAR subunits are synthesized and assembled in the rough endoplasmic reticulum (ER) and then inserted into the plasma membrane after crossing the Golgi apparatus. Vesicles are formed at the level of the ER and Golgi through complex coat protein-mediated membrane budding and fusion steps (Schekman and Orci, 1996). After being released from the Golgi, the secretory vesicles are trafficked to the plasma membrane, presumably through interactions with microtubules and microtubule-based motor proteins (Vallee and Sheetz, 1996). The secretory vesicles are inserted at the cell surface through SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) -dependent exocytosis (Lledo et al., 1998; Luscher et al., 1999), and AMPARs are removed from plasma membrane by clathrin-mediated endocytosis (Carroll et al., 1999; Man et al., 2000). Moreover, AMPARs on the cell surface move between synaptic and extrasynaptic membrane through lateral diffusion, and synaptic AMPARs are relatively immobile when compared with extrasynaptic AMPARs (Groc and Choquet, 2006).

Many studies have shown that the mechanisms regulating AMPAR exocytosis are subunit specific. The surface insertion of the GluR2 subunit is rapid and constitutive under basal conditions, without the need for synaptic activity (Passafaro et al., 2001; Shi
et al., 1999). In contrast, GluR1 exocytosis is slow in basal conditions, and is stimulated by neuronal activity and NMDAR activation (Hayashi et al., 2000). The native AMPARs consist mostly of GluR1/GluR2 or GluR2/GluR3 heteromers, together with a smaller contribution from GluR1 homomers (Wenthold et al., 1996). Based on experiments in which recombinant AMPA receptor subunits were expressed in hippocampal neurons, GluR1 is dominant over GluR2 in controlling the insertion of GluR1/GluR2 heteromers, whereas GluR2/GluR3 heteromeric receptors behave like GluR2 homomers and constitutively traffic into the synapses (Hayashi et al., 2000; Passafaro et al., 2001; Shi et al., 1999). So these subunit-specific rules lead to a simple model in which GluR2/GluR3 receptors traffic in and out of synapses constitutively, and GluR1 containing receptors are inserted into synapses in an activity-dependent manner during synaptic plasticity. However, whether the synaptic delivery of endogenous AMPARs followes the subunit-specific rules established from experiments with recombinant receptors still remains to be clarified.

Once inserted into the plasma membrane, the surface AMPARs rapidly move between extrasynaptic and synaptic plasma membrane through lateral diffusion (Adesnik et al., 2005; Groc et al., 2004). The trapping and anchoring of these wandering AMPARs at the postsynaptic membrane rely on PDZ-dependent interactions between postsynaptic density protein-95 (PSD-95) and transmembrane AMPA receptor regulatory proteins (TARPs) (Bats et al., 2007; Ziff, 2007). Furthermore, the surface AMPARs are internalized through clathrin-dependent endocytosis in an activity-dependent manner (Beattie et al., 2000; Man et al., 2000). In cultured neurons, several stimuli can cause AMPAR internalization, such as the activation of NMDARs (Beattie et al., 2000; Carroll
et al., 1999; Ehlers, 2000), AMPARs (Lin et al., 2000), mGluRs (Snyder et al., 2001; Xiao et al., 2001) or insulin receptors (Lin et al., 2000; Man et al., 2000). In contrast to the subunit-specific rules for AMPAR insertion, the contribution of different receptor populations to activity-dependent removal remains controversial. In fact, most experimental evidence is compatible with an initial indiscriminate internalization of both GluR1-containing and GluR2-containing AMPA receptor upon the stimuli (Meng et al., 2003; Seidenman et al., 2003). Moreover, the internalized AMPARs are differentially sorted in either the recycling endosomes that contain a pool of AMPARs for the rapid insertion, or degradative pathway (Ehlers, 2000; Lin et al., 2000).

It is becoming more evident that exocytosis, lateral diffusion and endocytosis are key players in AMPAR trafficking. Regulated insertion and removal of AMPARs in the synaptic membrane play key roles in the expression of LTP and LTD. The underlying mechanisms are still not clear, but more and more evidence suggest that phosphorylation/dephosphorylation and the interacting proteins modulate receptor trafficking.

1.2.2 AMPAR phosphorylation

The identification of the phosphorylation sites on the AMPAR has been the focus of many studies, as intracellular perfusions of cAMP-dependent protein kinase (PKA), protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaMKII) have been shown to potentiate AMPA receptor currents (Greengard et al., 1991; McGlade-McCulloh et al., 1993; Wang et al., 1994; Wang et al., 1991). Using site-directed mutagenesis and phosphopeptide mapping analysis, two major phosphorylation sites on the GluR1 C-terminal, Ser831 and Ser845, were identified (Roche et al., 1996).
PKA phosphorylates Ser845, whereas Ser831 is phosphorylated by both PKC and CaMKII (Barria et al., 1997; Mammen et al., 1997; Roche et al., 1996).

Electrophysiologically, phosphorylation of the GluR1 subunit on Ser845 increases the channel open probability (Banke et al., 2000) and the peak amplitude of the current (Roche et al., 1996). On the other hand, phosphorylation of Ser831 increases the single-channel conductance (Derkach et al., 1999). The activation of CaMKII, triggered by Ca\(^{2+}\) influx through the NMDARs, is believed to be essential for the induction of LTP (Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992). In addition, the intracellular perfusion of constitutively active CaMKII not only enhances the synaptic transmission, but also occludes LTP (Lledo et al., 1995). However, exogenous phosphorylation of the other phosphorylation site on GluR1, Ser 845, does not induce the delivery of recombinant GluR1 subunit. Also, Ser845 is thought to be phosphorylated by PKA under basal conditions and, therefore, may not participate in LTP (Chavez-Noriega and Stevens, 1994; Kameyama et al., 1998; Rosenmund et al., 1994). So it was of considerable interest to expect that phosphorylation of Ser831 is required for synaptic delivery of GluR1-containing receptors. However, mutations on GluR1-Ser831 that prevent its phosphorylation by CaMKII do not prevent delivery of the receptor to synapses by active CaMKII or by LTP (Hayashi et al., 2000). Interestingly, mutations at Ser845, the PKA phosphorylation site of GluR1, do prevent delivery of GluR1 to synapses by active CaMKII or LTP (Lee et al., 2003; Roche et al., 1996; Shi et al., 2001). Thus, CaMKII may act on some unknown targets to effect synaptic delivery of the GluR1 subunit during LTP. And phosphorylation of Ser845, although it is not sufficient, is necessary for GluR1 synaptic delivery.
A variety of studies have shown that protein phosphatases are crucial for NMDA-dependent LTD induction, suggesting that LTD is the functional inverse of LTP. It was, therefore, initially thought that LTD would promote the dephosphorylation of the CaMKII site of GluR1, Ser831. Surprisingly, however, LTD induces the dephosphorylation of the PKA site of GluR1, Ser845, with little effect on Ser831 phosphorylation (Lee et al., 2000; Lee et al., 1998). And LTD induction leads to dephosphorylation of Ser831 only in previously potentiated synapses (Lee et al., 2000). Furthermore, both LTP and LTD were diminished in a knock-in mouse in which both Ser831 and Ser845 were mutated (Lee et al., 2003), suggesting a role of the regulated phosphorylation of AMPAR subunits in synaptic plasticity.

In addition to the GluR1 subunit, GluR2 has also been shown to have two phosphorylation sites, Ser863 and Ser880, on its C-terminal domain (Chung et al., 2000; Matsuda et al., 1999; McDonald et al., 2001). Both Ser863 and Ser 880 are phosphorylated by PKC in vitro and in vivo. In addition, Ser880 is located in the region of the GluR2 C terminus that interacts with several intracellular PDZ domain-containing proteins (Chung et al., 2000; Matsuda et al., 1999). Interestingly, phosphorylation of GluR2 Ser880 disrupts the interaction with GRIP (glutamate receptor-interacting protein)/ABP (AMPA-binding protein) (Chung et al., 2000; Matsuda et al., 2000; Matsuda et al., 1999). Disruption of this interaction by Ser880 phosphorylation results in the removal of the receptor from the synapse by facilitating GluR2-PICK1 (protein interacting with C-kinase 1) interactions (Kim et al., 2001; Perez et al., 2001; Xia et al., 2000), which might mediate LTD.
Previous reports have focused on serine and threonine phosphorylation of AMPA receptors. However, recent evidence suggested that the tyrosine phosphorylation of the AMPARs also plays a role in the regulation of receptor functions. Although there is no tyrosine residues among the last 75 amino acids of the GluR1 C-terminal, nonreceptor Src family tyrosine kinases (Trks) have been shown to induce tyrosine phosphorylation of GluR1 (Moss et al., 1993; Rong et al., 2001). Unlike GluR1, the end region of GluR2 C-terminal has multiple tyrosine residues. And Src family Trks phosphorylate tyrosine 876 (Hayashi and Huganir, 2004), a last tyrosine residue near the end of C-terminal and within the C-terminal PDZ ligand domain. Phosphorylation of this site inhibits the association of the GluR2 subunit with GRIP/ABP, but not protein interacting with PICK1, and results in internalization of GluR2 (Hayashi and Huganir, 2004). Similar to Src, insulin and low-frequency stimulation (LFS) increase the phosphorylation of the last three tyrosine residues on the GluR2 C-terminal (Ahmadian et al., 2004). And truncation of the last 15 amino acids of the GluR2 C-terminal or point mutation of these three tyrosine residues in this region prevented the insulin-induced AMPAR endocytosis and LFS-induced LTD (Ahmadian et al., 2004). In addition, using a short synthetic GluR2_{3Y} peptide, which mimics the phosphorylation of the three tyrosine residues of the GluR2 C-terminal, blocked the insulin-induced AMPAR endocytosis in cultured hippocampal neurons, and inhibited the LFS-induced LTD in hippocampal slices (Ahmadian et al., 2004). Furthermore, GluR2_{3Y} peptide, which blocks activity dependent endocytosis of AMPARs, has been used as a specific inhibitor for LTD in many studies (Brebner et al., 2005; Kim et al., 2007; Wong et al., 2007).
1.2.3 AMPAR interacting proteins

Several AMPAR interacting proteins have been identified and been shown to play important roles in the modulation of AMPAR trafficking. TARPs are a family of transmembrane proteins sharing a weak homology with theγsubunits of muscle voltage-dependent Ca\(^{2+}\) channels (VDCCs) (Burgess et al., 1999; Klugbauer et al., 2000). TARPs coassemble with AMPARs early in the synthetic pathway and control their maturation, trafficking, and biophysical properties (Nicoll et al., 2006; Ziff, 2007). TARPs are also involved in folding and assembly of AMPAR, stabilizing, and facilitating their export from the ER (Tomita et al., 2003; Vandenberghe et al., 2005). Furthermore, TARPs promote surface expression of AMPARs (Chetkovich et al., 2002; Schnell et al., 2002; Tomita et al., 2003) and are critical for clustering AMPARs at excitatory synapses through their interaction with PSD-95 (Chen et al., 2000; Schnell et al., 2002), a major component of the postsynaptic scaffold (Kim and Sheng, 2004). In addition to the effect on AMPAR trafficking, TARPs control AMPAR channel gating by slowing glutamate-induced AMPAR deactivation and desensitization, and enhancing the channel conductance (Nicoll et al., 2006; Priel et al., 2005; Tomita et al., 2005).

In addition to TARPs, a recent study identified another auxiliary subunit of AMPARs, cornichon proteins (Schwenk et al., 2009). Cornichons increase surface expression of AMPARs in the heterogeneous expression system. Also, cornichons change the channel gating properties of AMPARs with a more significant effect in slowing deactivation and desensitization kinetics than TARPs (Schwenk et al., 2009). However, the functions of endogenous cornichons in the modulation of native AMPARs still need to be identified.
Besides indirect binding with PSD-95 through TARPs, AMPAR subunits have PDZ consensus motifs in the C-terminal that directly bind several PDZ domain-containing proteins. SAP97 (synapse-associated protein 97), a member of membrane-associated guanylate kinases (MAGUKs) family, binds to the PDZ domain of GluR1 subunit (Leonard et al., 1998; Rumbaugh et al., 2003), which seems to occur in the ER (Sans et al., 2001). The interaction with SAP97 might be involved in the CaMKII-mediated targeting of AMPARs to synapses (Hayashi et al., 2000). However, complete deletion of this PDZ domain of GluR1 does not appear to affect AMPA-receptor-mediated synaptic transmission or plasticity (Kim et al., 2005a). SAP97 also binds AKAP79 (A-kinase anchoring protein 79), which is a multivalent signaling scaffold that binds to PKA, calcineurin (pp2B) and PKC, and targets these kinases or phosphatases to GluR1 for phosphorylation or dephosphorylation (Colledge et al., 2000; Tavalin, 2008). The GluR2 C-terminal has a PDZ motif that interacts with several PDZ domain-containing proteins, GRIP, ABP, and PICK1, which play critical roles in AMPAR endocytosis and LTD. The binding of GRIP/ABP stabilizes GluR2-containing AMPAR at synapses by limiting their endocytosis or by increasing recycling of GluR2 (Osten et al., 2000). Studies of LTD in cerebellar Purkinje cells found that phosphorylation of Ser880 within the GluR2 PDZ-binding site prevents the association of GluR2 with GRIP and ABP, but promotes binding to PICK1, which mediates AMPAR endocytosis and LTD. GRIP/ABP also interacts with microtubule motor protein kinesin and promotes AMPAR dendritic transport (Setou et al., 2002). Besides interacting with GluR2/3 C-terminus, PICK1 also interacts with PKCα, and it has been proposed that dimeric PICK1 can act to chaperone activated PKC and AMPARs (Chung et al., 2000; Perez et al., 2001).
GluR1 also binds protein 4.1N, a member of multifunctional cytoskeletal components family, which links GluR1 to the actin cytoskeleton and stabilizes the surface expression of GluR1 (Shen et al., 2000).

The trafficking of AMPARs is also modulated by another GluR2 interacting protein, N-ethylmaleimide-sensitive fusion protein (NSF), which belongs to type II AAA (ATPase associated with a variety of activities) ATPase family. NSF interacts with a membrane proximal segment of the C-terminus of GluR2 (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998), and regulates rapid exocytosis of GluR2-containing AMPARs through its classical role in controlling membrane fusion (Rothman, 1994). Also, NSF helps to maintain the synaptic expression of GluR2-containing AMPARs (Hanley et al., 2002; Noel et al., 1999). Blocking these interactions with a peptide, ‘pep2m’ (KRMKVAKNAQ), causes a rapid run-down in AMPAR-mediated excitatory postsynaptic currents (EPSCs) at hippocampal CA1 synapses (Nishimune et al., 1998; Song et al., 1998), which is due to a loss of surface-expressed AMPARs (Luscher et al., 1999; Noel et al., 1999). NSF also displaces PICK1 from the PICK1-GluR2 complex and thereby facilitates the delivery or stabilization of GluR2 at the plasma membrane (Hanley et al., 2002). In the meanwhile, the clathrin adaptor protein AP2 interacts with a site that overlaps with NSF-binding site on GluR2, and is critical for NMDA-induced internalization of AMPARs (Lee et al., 2002). Moreover, using a peptide that specifically blocked the GluR2-AP2 interaction inhibited the induction of LTD (Lee et al., 2002). Since AP2 is directly involved in clathrin-dependent endocytosis, this provides a simple mechanism by which AMPARs are internalized during NMDA receptor-
dependent LTD, and the NSF-GluR2 interaction is primarily involved in the insertion or stabilization of AMPARs during basal synaptic transmission.

The extracellular N-terminal is the longest domain among the entire structure of GluR subunits, but less interacting proteins have been identified compared with other domains of GluR subunits. Recently, studies that tried to identify the N-terminal interacting partners demonstrate a role for AMPAR N-terminal protein-protein interactions either in receptor trafficking or in synaptogenesis. One type of the N-terminal interacting proteins that have been shown to be involved in the synaptic clustering of AMPARs are the neuronal pentraxins (O'Brien et al., 1999; Sia et al., 2007), including neuronal-activity-regulated pentraxin (Narp), neuronal pentraxin 1 (NP1), and neuronal pentraxin receptor (NPR) (Song and Huganir, 2002). Narp and NP1 coimmunoprecipitate with AMPARs in heterologous cells (Xu et al., 2003), and Narp-expressing HEK cells seeded on neurons recruit GluR1 to the sites where they contact neuronal dendrites (O'Brien et al., 1999). In cultured neurons, overexpression of Narp increases the number of synaptic AMPARs, and expression of dominant-negative forms of Narp decreases AMPAR clustering (O'Brien et al., 2002; O'Brien et al., 1999). Another N-terminal interacting protein, N-cadherin, a cell adhesion molecule, has been shown to directly bind to GluR2 N-terminal and involves in the formation and growth of dendritic spines in cultured hippocampal neurons (Saglietti et al., 2007). The extracellular N-terminal domain of GluR2 promotes the formation and growth of dendritic spines (Passafaro et al., 2003), and overexpression of that domain increases the frequency of miniature excitatory postsynaptic currents (mEPSCs) in cultured hippocampal neurons (Saglietti et al., 2007). On the other hand, RNAi knockdown of N-
cadherin prevents the enhancing effect of GluR2 on spine morphogenesis and mEPSC frequency (Saglietti et al., 2007). Thus, the GluR2NT and N-adherin interaction provides a structural connection between the postsynaptic AMPARs and the presynaptic terminals that stimulates presynaptic development and promotes dendritic spine formation.

1.2.4 Ca$^{2+}$-permeable AMPAR and synaptic plasticity

Until recently, our understanding of synaptic plasticity was based largely on studies of synapses that express mainly Ca$^{2+}$-impermeable AMPARs. However, more and more evidence has suggested the involvement of Ca$^{2+}$-permeable GluR2-lacking AMPARs in mediating synaptic plasticity, by offering a route for Ca$^{2+}$ entry independent of NMDARs or voltage-gated Ca$^{2+}$ channels. The first indication that novel forms of long-lasting synaptic plasticity could be observed by the activation of GluR2-lacking AMPARs came from studying excitatory transmission onto interneurons of the basolateral amygdale (Mahanty and Sah, 1998) and CA3 area of the hippocampus (Laezza et al., 1999; Lei and McBain, 2002). Repetitive activation of synaptic inputs onto interneurons of the amygdale produces LTP (Mahanty and Sah, 1998), which is crucial for enhanced synchronization of activity among neurons in response of fear conditioning (Quirk et al., 1995). Tetanic stimulation of mossy fiber inputs onto interneurons of the hippocampal CA3 area, which activates postsynaptic Ca$^{2+}$-permeable AMPARs, induces LTD (Laezza et al., 1999; Lei and McBain, 2002).

Long-lasting Ca$^{2+}$-permeable AMPAR-dependent synaptic plasticity is not only limited to changes in synaptic efficiency, but also involve activity-dependent changes in the subunit composition of AMPARs. For example, synaptic activity blockade modifies Ca$^{2+}$-permeability and subunit composition of postsynaptic AMPARs. In cultured
hippocampal neurons, chronic AMPAR blockade induces a switch from GluR2-containing to GluR1-dominated synaptic AMPARs (Ju et al., 2004; Thiagarajan et al., 2005). In contrast, potentiation of activity increases the proportion of GluR2-containing AMPARs in pyramidal cell spine heads in hippocampal slice cultures (Bagal et al., 2005). This form of homeostasis produces the basic regulation of synaptic strength in response to changes in synaptic transmission.

The best-characterized example of activity-dependent changes of AMPAR subunit composition is parallel fiber-stellate cell synapses in cerebellum. Ca$^{2+}$ entry through existing postsynaptic GluR2-lacking AMPARs (possibly GluR3 homomers) triggers a lasting switch to GluR2-containing AMPARs, a process involving the interacting protein partners for the delivery and anchoring of receptors (Gardner et al., 2005; Liu and Cull-Candy, 2005). The Ca$^{2+}$ influx induces dissociation of GRIP from GluR2-lacking AMPARs and retrieval of receptors from synaptic sites (Liu and Cull-Candy, 2005). In addition, Ca$^{2+}$ entry promotes the association of PICK1 and NSF with GluR2-containing receptors and facilitates their delivery to postsynaptic sites (Gardner et al., 2005). The rapid replacement of GluR2-lacking receptors by GluR2-containing receptors, results in a reduced EPSC, probably due to the lower single-channel conductance of GluR2-containing receptors compared with GluR2-lacking receptors (Cull-Candy et al., 2006).

Activity-dependent alterations in the composition of AMPAR also occur at synapses onto CA1 pyramidal neurons. Recent work by Isaac and co-workers showed in the Schaffer collateral-CA1 synapses, which express primarily Ca$^{2+}$-impermeable AMPARs, the induction of LTP causes transient synaptic incorporation of GluR2-lacking
(possibly GluR1 homeric) receptors (Plant et al., 2006). Ca^{2+} entry through these receptors is suggested to be important for the subsequent insertion of Ca^{2+}-impermeable AMPARs and thus, the full expression of LTP (Plant et al., 2006). Unlike the changes of AMPAR subunit composition in cerebellar stellate cells, the mechanisms underlying this process in hippocampal CA1 neurons are far from clear. One of the potential mechanisms is the interacting protein partners, which have been shown to play a role in the change of AMPAR subunit composition in cerebellar stellate cells. However, the difference between the subtypes of GluR2-lacking AMPARs in hippocampal CA1 neurons (GluR1 homomers) and in cerebellar stellate cells (GluR3 homomers) makes it a necessary step to identify new interacting partners for GluR2-lacking AMPARs in hippocampal neurons.
1.3 NMDAR and synaptic plasticity

Among the six regulatory subunits of NMDARs, NR2A and NR2B have been the most extensively studied because they are broadly expressed in the brain, predominate in the postnatal cortex, and are believed to play important roles in synaptic plasticity. NR2A and NR2B subtypes of NMDARs are present as either di-heteromers (NR1/NR2A or NR1/NR2B) or tri-heteromers (NR1/NR2A/NR2B). In addition, NR2A- and NR2B-containing NMDARs differ in channel kinetics, synaptic localization, and protein binding partners, all of which are expected to influence the induction of synaptic plasticity.

1.3.1 Characteristics of NR2A and NR2B subunits

NR1/NR2A di-heteromeric channels exhibit faster rising and decaying currents than NR1/NR2B di-heteromeric channels (Chen et al., 1999; Monyer et al., 1994; Prybylowski et al., 2002; Vicini et al., 1998). NR1/NR2A channels have higher open probability and faster deactivation than NR1/NR2B channels (Chen et al., 1999; Erreger et al., 2005). Although NR1/NR2B channels may have lower peak currents, they carry about two-fold more charge for a single synaptic event than NR1/NR2A channels, due to their slower deactivation which is enough to compensate for their lower open probability (Erreger et al., 2005). NR1/NR2A/NR2B tri-heteromeric channels appear to exhibit intermediate decay time courses between the two di-heteromeric channel types (Vicini et al., 1998).

NMDARs are located both at synaptic and extrasynaptic sites including the cell soma and dendritic shaft. Glutamate from spontaneous transmitter release reaches only the central part of synapses, while action potentials or repeated stimuli drive the spill over of glutamate, which can also reach extrasynaptic NMDARs. Synaptic and extrasynaptic
NMDARs are shown to couple to distinct intracellular signaling pathways (Ehlers, 2003; Hardingham et al., 2002; Ivanov et al., 2006), resulting in their different roles in mediating synaptic plasticity that synaptic NMDARs support LTP, while extrasynaptic NMDARs mediate LTD in the mature brain (Lu et al., 2001b; Massey et al., 2004), although this remains controversial. The possibility that the subunit composition of NMDARs differs between synaptic and extrasynaptic sites is also controversial. It has been proposed that NR2A-containing NMDARs are more likely to occupy the central portion of the synapse, while NR2B-containing NMDARs are preferentially targeted to peripheral portions of the synapse or to extrasynaptic sites. This view has arisen from the finding that, in rat dentate gyrus granule cells, NMDAR-mediated mEPSCs exhibit faster decay kinetics than evoked NMDAR-mediated EPSCs (Dalby and Mody, 2003). Moreover, spontaneous neurotransmitter release fails to activate NMDAR currents in the midbrain of NR2A knockout mice, while evoked synaptic activity can drive NMDAR currents in the absence of NR2A (Townsend et al., 2003; Zhao and Constantine-Paton, 2007). While some studies suggest that NR2B-containing NMDARs are most prevalent at extrasynaptic sites (Scimemi et al., 2004; Stocca and Vicini, 1998; Tovar and Westbrook, 1999), other studies suggest that both NR2A- and NR2B-containing NMDARs exist extrasynaptically (Mohrmann et al., 2000) and that the ratio of the two subtypes is comparable to that of synaptic NMDARs (Thomas et al., 2006). Some of these apparent discrepancies may be resolved by examining developmental and regional differences in the localization of NMDAR subtypes (Kohr, 2007).
1.3.2 Interacting proteins of NR2A and NR2B subunits

The NMDAR-dependent LTP and LTD are dependent on the downstream signaling pathways which tightly couple to NMDARs (Kennedy et al., 2005). NR2A and NR2B subunits each interact with different intracellular proteins, which are important in determining the direction of synaptic plasticity. For example, NR2B interacts directly with Ras-guanine nucleotide-releasing factor 1 (Ras-GRF1), a calcium sensor that activates NMDAR-dependent ERK pathway (Krapivinsky et al., 2003). Ras-GRF1 mediates signaling from NR2B-containing NMDARs to the Ras effector p38 MAP kinase, a promoter of LTD (Li et al., 2006). In contrast, Ras-GRF2 mediates signaling from NR2A-containing NMDARs to the Ras effector extracellular signal-related protein kinase 1/2 (Erk1/2) mitogen-activated protein (MAP) kinase, a promoter of NMDAR-induced LTP (Li et al., 2006), although the direct binding between NR2A and Ras-GRF2 has not been reported.

Both NR2A and NR2B interact with membrane-associated guanylate kinase (MAGUK) family of synaptic scaffolding proteins, which are important for the dendritic transport, synaptic localization, and trafficking of receptors (Wenthold et al., 2003). NR2A preferentially binds to PSD-95, while NR2B preferentially binds synapse-associated protein 102 (SAP102) (Sans et al., 2000; Townsend et al., 2003). Moreover, these interactions are thought to control distinct synaptic localization of NR2A and NR2B (Townsend et al., 2003). During development, the overall expression of PSD-95 is increased, favoring an increase in NR2A and a decrease in NR2B at the synapse (Sans et al., 2000). These synaptic scaffolding proteins also associate with important synaptic signaling molecules, and thus mediate signaling from NMDARs to intracellular pathways.
(Kennedy, 2000). For example, NR2B is linked indirectly to synaptic Ras GTPase activating protein (RasGAP), presumably through SAP102 (Kim et al., 1998). In addition, the unique association of NR2B to Ras-GRF1 plays a critical role in the regulation of neuronal MAPK signaling, AMPAR membrane trafficking, and excitatory synaptic transmission (Rumbaugh et al., 2006).

One of the most important NMDAR binding partners is CaMKII, which translocates to synapses (Otmakhov et al., 2004; Shen and Meyer, 1999; Strack et al., 1997) and binds to NMDARs (Bayer et al., 2001; Leonard et al., 1999; Strack et al., 2000) upon activation by Ca\(^{2+}\)/calmodulin. CaMKII binds with a high affinity to NR2B subunit (Leonard et al., 1999; Strack and Colbran, 1998; Strack et al., 2000), and to a much lesser extent to NR2A subunit (Gardoni et al., 1999; Strack and Colbran, 1998). Given the fact of the synaptic location of NR2A, binding of CaMKII to NR2B may permit cooperative functions between NR2A and NR2B, which is required for plasticity (Kim et al., 2005b). The initial CaMKII-NR2B interaction requires the activation of CaMKII which is dependent on Ca\(^{2+}\) influx through NMDARs followed by activation of the Ca\(^{2+}\) binding protein, calmodulin. Activated CaMKII binds strongly to NR2B (Strack and Colbran, 1998), allowing CaMKII to remain active even after dissociating from Ca\(^{2+}\)/calmodulin (Bayer et al., 2001). Moreover, NR2 subunits can regulate synaptic plasticity through their different affinity for activated CaMKII, and the association between active CaMKII and NR2B is required for different forms of synaptic enhancement (Barria and Malinow, 2005). The decreased synaptic plasticity is observed in association with the switch from synaptic NR2B-containing receptors to NR2A-containing receptors during development.
(Carmignoto and Vicini, 1992; Monyer et al., 1994), which is probably due to the decreased association of active CaMKII with synaptic NMDARs.

1.3.3 Roles of NR2A and NR2B in LTP and LTD

Given the facts of the distinct gating and pharmacological properties and synaptic or extrasynaptic distribution of the different NMDAR subtypes, it is proposed that the activation of NR2A- and NR2B-containing NMDARs may determine the direction of synaptic plasticity that leads to LTP and LTD. Using specific antagonists for NR2A- (NVP-AAM077) and NR2B-containing (ifenprodil and Ro25-6981) NMDARs, the study in the hippocampus slices showed that selectively blocking NMDARs that contain the NR2B subunit abolishes the induction of LTD but not LTP. In contrast, preferential inhibition of NR2A-containing NMDARs prevents the induction of LTP without affecting LTD production (Liu et al., 2004a). The idea that NR2A-containing NMDARs mediate LTP and NR2B-containing NMDARs mediate LTD has been supported by other studies. For example, in the adult perirhinal cortex, similar results were observed that LTD requires the activation of NR2B-containing NMDARs, whereas LTP requires activation of NR2A-containing NMDARs (Massey et al., 2004). An in vivo study in the anesthetized rats, which was designed to identify the involvements of NR2A and NR2B in LTP and LTD, found blocking LTP and LTD by their antagonist is dependent on the dose. Ro25-6981 (6 mg/kg, i.p.) and NVP-AAM077 (1.2 mg/kg, i.p.) specifically interfered with LTD and LTP, respectively. However, a higher dose of either antagonist affected both LTP and LTD (Fox et al., 2006). Moreover, in NR2A knockout mice, NMDAR- and L-type Ca^{2+} channel-dependent LTP is absent in juvenile superior
colliculus, whereas low frequency-induced LTD is intact, which can be blocked by Ro 25-6981 (Zhao and Constantine-Paton, 2007).

However, mediation of LTP by NR2A-containing NMDARs has been challenged because of the specificity of NVP-AAM077. Unlike the high selectivity of ifenprodil and Ro25-6981 for NR1/NR2B channels, NVP-AAM077 is 10-100 times more selective for NR1/NR2A channels than NR1/NR2B channels (Liu et al., 2004a). Controversy remains regarding the specific role of NR2B-containing receptor in LTD. It has been reported that NR2B-containing, but not NR2A-containing, NMDARs mediate LTP in studies done in immature hippocampal slice cultures using a pairing protocol (Barria and Malinow, 2005), in the anterior cingulate cortex of six- to eight-week-old mice by either a pairing induction protocol or theta-burst stimulation (Zhao et al., 2005), and in thalamocortical synapses in the barrel cortex of postnatal day 3-5 mice (Lu et al., 2001a). In addition, hippocampal LTD was also reported to be insensitive to ifenprodil (Morishita et al., 2007), and even enhanced by ifenprodil (Hendricson et al., 2002). The controversial nature of these findings may be explained by the different developmental and regional distribution of NR2A- and NR2B-containing NMDARs. Moreover, signaling molecules downstream of NMDARs vary with region and developmental stage (Chattopadhyaya et al., 2004; Jiang et al., 2005), which are also important contributors to the induction of plasticity (Choi et al., 2002; Steele and Mauk, 1999). Another consideration of these pharmacological studies is the complex nature of ifenprodil (Neyton and Paoletti, 2006), which blocks NMDARs at high concentrations of glutamate and potentiates NMDAR currents at low glutamate concentrations (Kew et al., 1996). Thus, ifenprodil may affect synapses differently depending on the glutamate concentration in the synaptic cleft.
Moreover, in the adult perirhinal cortex, the ifenprodil-dependence of LTD relies on the state of the synapse; ifenprodil blocks LTD that has been induced at a basal state, but fails to block depotentiation (a form of LTD induced at recently potentiated synapses) (Massey et al., 2004).
1.4 Synaptic plasticity and spatial memory

Learning may be described as the mechanism by which new information about the world is acquired, and memory as the mechanism by which that knowledge is retained. Memory is categorized as explicit memory, which is defined as that involved in the conscious recall of information about people, places, and things; or implicit memory, which is characterized by the nonconscious recall of tasks such as motor skills. One of the most compelling problems in neuroscience is to identify the mechanisms underlying memory. Although a great deal of progress has been made in the past few decades, it remains a significant challenge. Particular emphasis has been placed on analysis of changes that accompany and support spatial memory because of the well-developed protocols that are available for its analysis. In addition, the use of innovative lesion techniques, localized pharmacological treatments, and molecular genetic interventions is offering increasing evidence that spatial memory is dependent on hippocampus and synaptic plasticity plays an important role in it.

1.4.1 Synaptic plasticity and memory (SPM) hypothesis

In 1949, Hebb proposed the following synaptic learning rule (Hebb, 1949):
‘When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficacy, as one of the cells firing B, is increased.’ This learning rule has been popularly reinterpreted as ‘neurons that fire together wire together’. In the last 35 years or so, great strides have been made in uncovering the molecular mechanisms of learning and memory. Changing in the strength of connections between neurons is widely believed to be the mechanism by which memory traces are encoded and stored in
the central nervous system (Kandel and Schwartz, 1982; Morris and Frey, 1997). In addition, studies of the activity-dependent synaptic plasticity in the multiple types of memory has lead to a more specific hypothesis, called the synaptic plasticity and memory (SPM) hypothesis (Martin et al., 2000): ‘Activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed.’ This hypothesis is rooted in the fact that synaptic plasticity is a physiological phenomenon whereby specific patterns of neural activity give rise to changes in synaptic efficacy and neural excitability that long outlast the events that trigger them.

1.4.2 Hippocampus and spatial memory

Although several areas of the brain play a part in mediating several forms of learning and memory, the hippocampus has been recognized as playing a vital role in formation of explicit memory. The role of the hippocampus has attracted people’s attention since reports were published by Milner and colleagues concerning a human patient H.M., who as a young man suffered from intractable epilepsy and underwent experimental surgery involving bilateral removal of the medial temporal lobe (MTL), including large parts of both hippocampi. The procedure left H.M. with an inability to form new episodic memories (anterograde amnesia), coupled with a substantial, but not total, loss of old memories (retrograde amnesia) (Scoville and Milner, 1957). Subsequent studies on H.M. and other patients, as well as studies on animal models, have enriched our knowledge about the roles of the MTL and its components in declarative memory (Jarrard, 1993; Rempel-Clower et al., 1996; Schmolck et al., 2002; Zola and Squire,
Spatial memory, a type of declarative memory that is concerned with locations of specific objects and places within a spatial environment, has been studied extensively using the Morris water maze (MWM) in which an animal’s capacity to remember spatial cues is required to locate a hidden underwater platform (Morris et al., 1982). It has become clear that the hippocampus has an essential role in spatial memory (Morris et al., 1982; Silva et al., 1998). Rats with lesions, pharmacological inactivation, or molecular knockouts limited to the hippocampus result in either a failure to learn or a loss of spatial memory (Morris et al., 1986; Pastalkova et al., 2006; Tsien et al., 1996). The impairment of the spatial memory by the inactivation of hippocampus is not due to its influence on the visual or sensorimotor functions, because in the case of the Morris water maze, although lesioned rats perform very poorly when the platform is not visible, lesioned and nonlesioned rats perform in a comparable manner when the platform is visible. In addition, several studies, which were built on the original hypothesis of O'Keefe that the hippocampus encodes maps of space in a literal Euclidean sense, have lead to the discovery of hippocampal place cells (O'Keefe and Dostrovsky, 1971). Single-unit recordings from neurons in the hippocampus of freely moving rodents reveal that pyramidal and granule cells show a preference for firing in a particular location of an explored environment, regardless of the direction from which the animal enters the location (O'Keefe and Dostrovsky, 1971). It appears that the key role of the hippocampus in spatial learning is synthesis of the configuration of spatial cues, which is governed, at least to some extent, by temporal events (Teng and Squire, 1999). Significantly, other forms of learning, such as visual discrimination and taste aversion, are not affected by hippocampal lesions.
1.4.3 Hippocampal LTP and spatial memory

It is widely accepted that memory formation is dependent on changes in synaptic efficiency that permit strengthening of associations between neurons, and activity-dependent synaptic plasticity at appropriate synapses is believed to be both necessary and sufficient for storage of information. Following the Hebb’s hypothesis that if two neurons are active at the same time, the synaptic efficiency of the appropriate synapse will be strengthened, people have made an enormous effort to find mechanisms by which strengthening of synaptic connections can be achieved. The most important model is LTP, which was firstly discovered by Bliss and Lomo in rabbit hippocampus perforant path to dentate gyrus granule cells synapses (Bliss and Lomo, 1973). The three well-described characteristics of LTP, cooperativity, associativity and input specificity (Bliss and Collingridge, 1993), and long-lasting duration of LTP (Abraham et al., 1995), have been identified as solid arguments that support the hypothesis that LTP may be a biological substrate for at least some forms of memory. Several other pieces of evidence have supported this view. LTP is easily demonstrable in the hippocampus, an area of the brain known to be fundamentally important in memory acquisition. Theta burst stimulation, that mimics naturally occurring theta rhythm recorded in the hippocampus during exploratory behavior, can induce LTP (Diamond et al., 1988; Larson et al., 1986; Pavlides et al., 1988; Rose and Dunwiddie, 1986). Several biochemical changes that occur after induction of LTP also occur during memory acquisition (see below). Inhibitors of hippocampal LTP also block hippocampal learning and retention of tasks (Morris et al., 1986).
In addition, the character of the long-lasting duration also makes LTP an ideal cellular mechanism for maintenance of long-term memory in the brain. Memories can persist for varying periods of time, up to nearly a lifetime. Studies of hippocampal LTP in rats have demonstrated that LTP can be maintained across a time period similar to that for memories putatively stored in rodent hippocampus. At the cellular level, the storage of long-term memory is associated with gene expression, de novo protein synthesis, and formation of new synaptic connections. Protein synthesis inhibitors that have no effects on short-term memory block persistent long-term memory. There is an interesting parallel between memory and LTP, since it has been revealed that the early phase LTP, which lasts 2-3 hours, is independent of protein synthesis, while more persistent long-lasting LTP, which lasts several hours in vitro and weeks in vivo, required synthesis of new proteins (Krug et al., 1984). Moreover, the establishment of long-term memory shares cellular mechanisms with LTP, including dependence on early activation of the transcription (Bourtchuladze et al., 1994; Jones et al., 2001), and synaptic morphological changes such as increase in spine number and density, and synapse-to-neuron ratio (Eyre et al., 2003; Leuner et al., 2003; O'Malley et al., 2000).

Early work in support of the necessity of hippocampal LTP in spatial memory came from Morris and colleagues. They found that intraventricular infusion of the selective NMDAR blocker APV (2-amino-5-phosphonovaleric acid) profoundly impaired spatial learning on the hidden-platform MWM task, but not if the platform was visible (Morris et al., 1986). The estimated extracellular concentrations of APV in the hippocampus that caused the spatial learning impairment were comparable to those that blocked LTP in vivo at synapses between the perforant path and the dentate gyrus without
affecting basal synaptic transmission (Davis et al., 1992). In a recent experiment, it was shown that inhibition of the active form of the protein kinase PKMzeta by infusion into the hippocampus of its specific inhibitor ZIP (myristoylated zeta-pseudosubstrate inhibitory peptide), can impair spatial memory and the maintenance of LTP, even when the inhibitor is administered days after the acquisition of the memory or the induction of LTP (Pastalkova et al., 2006). However, in both cases it is impossible to be certain that the drug has not spread outside the hippocampus and is not having some effect other than blocking the induction or maintenance of LTP (Morris et al., 1986). So these findings suggested an overlap in the mechanism by which LTP was sustained and by which spatial learning was consolidated, but the necessity of LTP for spatial memory still cannot be concluded. A complementary approach was provided by genetically engineered mice, in which the NMDA receptor NR1 was reversibly inactivated in the CA1 subfield of the hippocampus (Shimizu et al., 2000). In this mouse both LTP and spatial learning are suppressed, implying that the presence of NR1 receptors in area CA1 is necessary for spatial learning to occur. However, the deletion of NR1 in CA1 also affects several other processes, such as certain forms of LTD (Dudek and Bear, 1992), and reduces postsynaptic responses during short bursts of high-frequency activation (Herron et al., 1986). Thus, definitive evidence is still needed to conclude that LTP is necessary for hippocampus-dependent spatial learning.

More recent studies have shown that inhibition of the NMDA receptor only impairs spatial memory in task-naive animals, whereas pretraining in a spatial task overcomes the inhibition induced by APV (Bannerman et al., 1995). In a ‘upstairs/downstairs’ water maze experiment, rats were trained in one maze (on a lower
floor of the laboratory building) and subsequently were able to learn and retain information about the location of the hidden platform in a second upstairs maze, even when infused with the NMDAR antagonist APV (Bannerman et al., 1995). This experiment suggests that, at least in some circumstances, conventional NMDAR-dependent LTP is not required for the acquisition and storage of hippocampus-dependent spatial memory. Another potential example for the learning without LTP is the GluR1-knockout mouse, which showed a total absence of conventional tetanus-induced LTP in area CA1 without any impairment in acquisition or recall in the standard Morris water maze (Zamanillo et al., 1999). However, subsequent analysis revealed that LTP could be induced using a theta-burst pairing protocol, in which presynaptic stimulation at 5 Hz was paired with synchronous depolarization of the CA1 pyramidal cell (Hoffman et al., 2002).

To test the sufficiency of hippocampal LTP for spatial memory, an experiment could involve artificially induce LTP at an appropriate subset of hippocampal synapses and determine whether this manipulation can achieve an apparent memory of an event that never occurred. However, this is unlikely to be feasible in the near future using rodents. Nevertheless, it may be feasible to employ this strategy in a simpler vertebrate system. For example, in the case of the goldfish escape reflex, it is known that repeated acoustic stimuli induce LTP at inhibitory synapses onto the Mauthner cell and that they also cause behavioral desensitization of this reflex (Oda et al., 1998).

In summary, there are some correlative evidence available to support a critical role of hippocampal LTP for the spatial memory. However, definitive experiments still need to be designed to prove whether LTP represents a biological substrate for leaning and memory.
1.4.4 Hippocampal LTD and spatial memory

Hippocampal LTD, like LTP, is also closely associated with spatial memory processing. Traditionally, LTP has been designated as the main mediator of spatial memory storage in the hippocampus, whereas LTD has been assigned an auxiliary role in creating a higher signal-to-noise ratio (Dayan and Willshaw, 1991) or in erasing stored information (Tsumoto, 1993). The first indication of a strong correlation between spatial memory and LTP was the experiment that spatial learning in the Morris water maze was impaired by NMDAR antagonist APV that also prevented hippocampal LTP (Morris et al., 1986). However, the involvement of LTD in spatial memory cannot be excluded, since the concentration of NMDAR antagonist that blocked LTP also completely blocks LTD (Manahan-Vaughan, 1997). Other evidence to correlate LTP to spatial memory was provided by genetically modified mice, in which the NMDA receptor NR1 was reversibly inactivated in the CA1, and both LTP and spatial learning are suppressed (Shimizu et al., 2000). However, the deletion of NR1 in CA1 also affects certain forms of LTD (Dudek and Bear, 1992). So these studies, which support a role of LTP in the spatial learning and memory, also imply that spatial learning requires LTD-like changes just as much as LTP-like changes. Actually, LTD can be induced by various stimuli that result in information storage. And LTD also fulfils the criteria that are believed to be necessary for memory formation: cooperativity, associativity and input specificity (Braunewell and Manahan-Vaughan, 2001; Dudek and Bear, 1992; Manahan-Vaughan and Braunewell, 1999; Xu et al., 1997). Given that all synapses studied in the brain to date can undergo LTP and LTD, it has been proposed that bidirectional nature of synaptic plasticity is an important clue to an understanding of memory formation (Bear and Abraham, 1996). Furthermore,
subsequent studies showed different roles of LTP and LTD in the spatial information processing.

The first indication that LTD might have a role in spatial memory processing derived from studies that examined whether presenting naive animals with a novel environment led to changes in synaptic plasticity (Manahan-Vaughan and Braunewell, 1999). Several experiments, employing in vivo electrophysiological recording in freely moving rats, have revealed that novel empty space reinforces the expression of LTP but impairs LTD in the CA1 region (Kemp and Manahan-Vaughan, 2004), whereas an environment that contains novel objects facilitates the expression of LTD but impairs LTP (Kemp and Manahan-Vaughan, 2004, 2005; Lemon and Manahan-Vaughan, 2006; Manahan-Vaughan and Braunewell, 1999). In another study in hippocampal brain slices taken from rats exposed to an enriched environment, LTP and LTD were both enhanced in CA1 region after 3-5 weeks (Artola et al., 2006). Taken together, these data suggest that LTD is involved with processing spatial information concerning object-place configuration (Kemp and Manahan-Vaughan, 2004).

More recent evidence supporting the role of LTD in spatial memory processing derived from the genetically modified mice in which serum response factor (SRF) was deleted (Etkin et al., 2006). These SRF knockout mice show normal hippocampal LTP but impaired LTD, and learn the non-hippocampus-dependent tasks, such as olfactory habituation and the acoustic startle reflex as well as control mice. However, the SRF knockout mice are unable to learn any of the hippocampus-dependent spatial learning tasks, such as contextual habituation and the water maze (Etkin et al., 2006), offering further support for a role of LTD in the acquisition of spatial context.
Another notable observation is that behavioural stress, which causes cognitive deficits, can facilitate and impair the induction of LTD and LTP, respectively, in the adult rat hippocampus (Diamond et al., 2005; Xu et al., 1997). This effect is robust and durable enough to be observed in hippocampal slices taken from rats 7-9 months after exposure to behavioural stress (Artola et al., 2006). The mechanisms for this remain to be established but stress and glucocorticoids elevate extracellular glutamate levels in the hippocampus by depressing the glutamate uptake mechanisms, resulting in the activation of extrasynaptic NR2B-containing NMDARs and, thus, facilitation of LTD (Yang et al., 2005). Previous work from our lab (Wong et al., 2007) found that acute stress, which facilitates LFS-induced hippocampal LTD in CA1 region, also disrupts the retrieval of spatial memory. Using two selective inhibitors, which target the induction or expression of LTD, also blocks spatial memory retrieval impairments caused by acute stress. So LTD is an essential requirement for stress-induced impairment of spatial memory retrieval.

In summary, the entire spectrum of hippocampus-dependent memories that can be encoded by LTD has yet to be identified. But one theory proposed to account for the above findings suggests that hippocampal LTD is of crucial importance for the novelty exploration and response to stress.
1.5 p97/VCP

p97, also called valosin-containing protein (VCP), is a member of the type II AAA ATPase family, which is characterized by the presence of two conserved ATPase domains (Confalonieri and Duguet, 1995; Neuwald et al., 1999). p97 was originally cloned as a precursor protein for a 25 amino acid peptide, valosin (Koller and Brownstein, 1987), which was isolated from pig intestine (Gill et al., 1989; Schmidt et al., 1985) and shown to affect gastro-intestinal activity in dogs (Konturek et al., 1987). Subsequently, p97 was rediscovered as a ubiquitous ATPase in Xenopus extracts and named p97 for its relative molecular mass in sucrose gradient sedimentation (Peters et al., 1990). p97 is an essential protein, conserved throughout evolution from archaea to mammals (Dreveny et al., 2004). In addition, p97 is highly abundant in cells, accounting for more than 1% of the total cellular proteins (Wang et al., 2004). EM studies showed that p97 is a homo-hexamer with 6 subunits arranged in a ring with a pore in the centre (DeLaBarre and Brunger, 2003; Zhang et al., 2000). Each subunit is composed of an N-terminal domain, two AAA domains which are responsible for nucleotide binding and hydrolysis, and a C-terminal domain (DeLaBarre and Brunger, 2003; Zhang et al., 2000). Similar to other AAA proteins, p97 catalyzes ATP hydrolysis to generate energy and uses the energy to perform mechanical work in cells (Wang et al., 2004). Moreover, p97 acts as a molecular chaperone that binds to adaptor proteins and transfers energy from ATP hydrolysis through the adaptor to mediate many cellular activities. Because of its involvement in an unusually wide variety of functions, p97 has attracted a great deal of attention from researchers in different fields. The involvement of p97 in neuronal activities has not been well studied, even though several lines of evidence have shown that p97 functions as an
effector for several neurodegenerative disorders (Hirabayashi et al., 2001; Kobayashi et al., 2002).

1.5.1 p97 structure

The p97 molecule can be divided into N (1–187), D1 (209–460), D2 (481–761), and C (762–806) domains, with the N–D1 linker (188–208) and D1–D2 linker (461–480) joining the respective domains (DeLaBarre and Brunger, 2003; Zhang et al., 2000). EM studies showed that p97 proteins form a barrel-like, homo-hexameric structure that consists of two ring-shaped layers made of the respective D1 and D2 AAA modules (Peters et al., 1992; Rouiller et al., 2000; Zhang et al., 2000). In addition, Cryo-EM analyses have shown that during the ATPase cycle, D1 and D2, as well as the N domain experience conformational changes in ATP binding, ATP hydrolysis, P, release, and ADP release (Rouiller et al., 2002).

The N domain is the least conserved region among AAA family proteins, and is expected to determine the specificity of binding target proteins. Indeed, the N domain of p97 is mostly flexible, and has been shown to be responsible for binding to the cofactors/adaptors including p47 and the Ufd1–Npl4 complex (where Ufd1 stands for ubiquitin fusion degradation 1 and Npl4 for nuclear protein localization 4) (Meyer et al., 2000), and the ubiquitinated target proteins, especially those conjugated with poly-Ub chains (Dai and Li, 2001). p97 forms hexameric structures in a nucleotide independent manner (Wang et al., 2003a; Wang et al., 2003b), and both crystallography and biochemical studies indicated that D1 is the major domain responsible for the oligomerization (Wang et al., 2003a; Zhang et al., 2000). Both D1 and D2 domains contribute to the ATPase activity of p97, and share a high sequence similarity. However,
in vitro assays showed that, at physiological temperatures, mutations in D2 significantly inhibit the ATPase activity, while similar mutations in D1 have less effect (Song et al., 2003; Ye et al., 2003). Therefore, D1 and D2 of p97 perform different functions: while D1 is responsible for oligomerization, D2 accounts for the major enzyme activity. Compared with the rest of the p97 molecule, the C-terminal is a highly flexible domain (Wang et al., 2003b), and is undetectable in crystallography and EM analyses. One striking characteristic of the C domain is the presence of tyrosine phosphorylation site, Tyr805, at the end of C-terminus (Egerton and Samelson, 1994), and the phosphorylation of this site has been shown to affect transitional ER assembly (Lavoie et al., 2000). Furthermore, the entire molecule of p97 is required for its biological functions (Song et al., 2003).

1.5.2 Diverse biological functions of p97

As mentioned above, p97 acts as a molecular chaperone in many seemingly unrelated cellular activities, such as membrane fusion, cell cycle regulation, stress response, programmed cell death, transcription activation, ER-associated degradation, and protein degradation (Dreveny et al., 2004; Wang et al., 2004). A plethora of adaptors have been reported for p97, and increasing evidence is suggesting that it is through adaptor binding that p97 is diverted into different cellular pathways (Yeung et al., 2008). p47 was first found as an essential adaptor protein for the p97-mediated membrane fusion pathway (Kondo et al., 1997). p47 mediates the interaction between p97 and syntaxin5, a membrane-localized p47 receptor and a t-SNARE (target-soluble NSF attachment protein receptor) family member (Rabouille et al., 1998). This complex is essential for the postmitotic membrane fusion processes which reconstitute ER and Golgi apparatus.
Recent evidence further suggested that another adaptor protein, VCIP135 (VCP-p47 complex-interacting protein, p135), is also required in this process (Uchiyama et al., 2002).

One of the most studied adaptors of p97 is the Ufd1-Npl4 (UN) complex (Hetzer et al., 2001; Meyer et al., 2000; Meyer et al., 2002; Ye et al., 2003). p97-UN complex is required in ER-associated degradation (ERAD), a process that misfolded and unassembled polypeptides that cannot reach their native conformation in the ER are translocated into the cytosol, conjugated with polyubiquitin and subsequently degraded by the 26S proteasome. p97 acts as a molecular chaperone that extracts the ubiquitinated proteins from the ER membrane before their final degradation by the proteasome (Bays et al., 2001; Hitchcock et al., 2001; Jarosch et al., 2002; Rabinovich et al., 2002; Rape et al., 2001; Ye et al., 2001). Moreover, p97 has been shown to directly bind polyubiquitinated proteins, as well as 26S proteasome (Dai et al., 1998; Ghislain et al., 1996; Kaneko et al., 2003; Koegl et al., 1999; Meyer et al., 2000; Verma et al., 2000). In addition, cells expressing ATPase-deficient VCP-K524A are characterized by accumulation of polyubiquitinated proteins in membrane fractions but not in cytosolic fractions, as well as decreased degradation of the ERAD substrate (Kobayashi et al., 2002).

Apoptosis is a programmed cell death that is essential for the development and homeostasis of all multicellular organisms. The finding that p97 acts as a chaperone in the Ubquitin-Proteasome pathway and the revelation that Ubquitin-Proteasome pathway participates in the apoptotic process (Jesenberger and Jentsch, 2002; Varshavsky, 2003; Yang and Yu, 2003), suggest an involvement of p97 in apoptosis. Indeed, several lines of evidence have shown that p97 is an anti-apoptotic agent. The initial study of
mammalian p97 in apoptosis was done in a cytokine stimulation model. p97 has been shown to be a target gene that is specifically expressed in stimulated murine cells expressing the cytoplasmic serine/threonine kinase Pim-1, a protein involved in gp130/STAT3-mediated cell survival and cell proliferation (Shirogane et al., 1999). Overexpression of p97 was able to partially replace Pim-1 in its role in promoting cell survival (Shirogane et al., 1999). In contrast, expression of mutant p97 with critical mutations in the conserved ATP binding sites resulted in a drastic decrease in cell proliferation and massive induction of apoptotic cell death (Shirogane et al., 1999).

Studies of morphological alterations and the induction of cell death in neuronally differentiated PC12 (rat pheochromocytoma) cells expressing p97 mutations also revealed an anti-apoptotic effect of p97 (Hirabayashi et al., 2001). The second ATPase domain of p97 (D2) is critical for cell survival, as deletion of the D2 domain, mutation of the conserved ATP binding (lysine 524 to alanine, K524A) or ATPase active site (glutamate 578 to glutamine, E578Q) of this domain resulted in cytoplasmic vacuoles, followed by cell death (Hirabayashi et al., 2001). Furthermore, the apoptotic cell death in cells expressing ATPase-deficient p97-K524A is associated with ERAD dysfunction, induction of ER stress, and abnormal ER expansion (Kobayashi et al., 2002). These data suggest that p97 mutations in the second ATPase domain (D2) trigger cell death via an ER stress-mediated apoptotic pathway.

Mutations in human p97 trigger the human multisystem disorder ‘inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia’ (IBMPFD) (Forman et al., 2006; Guinto et al., 2007; Guyant-Marechal et al., 2006; Haubenberger et al., 2005; Hubbers et al., 2007; Kimonis and Watts, 2005; Krause et al.,
2007; Schroder et al., 2005; Watts et al., 2007; Watts et al., 2004). This disorder is characterized by inclusion bodies in muscle or brain (Kimonis and Watts, 2005; Schroder et al., 2005; Watts et al., 2004). Most patients suffer from a strong myopathy that may be associated with bone disease and/or frontotemporal dementia (Kimonis and Watts, 2005; Watts et al., 2004). In addition, p97 also plays a role in several neurodegenerative disorders with intracellular protein inclusions such as polyglutamine in Parkinson’s and Alzheimer’s diseases (Hirabayashi et al., 2001; Ishigaki et al., 2004; Mizuno et al., 2003). Wild-type p97 co-localizes with protein aggregates (Hirabayashi et al., 2001; Ishigaki et al., 2004; Mizuno et al., 2003), and is involved in the processes of clearing the pre-formed aggresomes and expanded polyglutamine repeat aggregates (Kobayashi et al., 2007). Cells expressing p97 mutations showed an increased formation of both aggresome and expanded polyglutamine aggregates when compared with cells expressing wild-type p97 (Kitami et al., 2006; Kobayashi et al., 2007).

Similar to p97 mutations, depletion of p97, which also decreases cellular activities of p97, results in reduced cell proliferation and triggers apoptotic cell death (Wojcik et al., 2004). Applying an RNA interference (RNAi) approach in human HeLa cells, Wojcik and colleagues demonstrated that depletion of p97 affected ubiquitin-dependent protein degradation, leading to the cellular accumulation of polyubiquitinated proteins (Wojcik et al., 2004). Taken together, the anti-apoptotic role of p97 has made it an essential protein for cell survival, and either mutation or deletion of p97 results in the accumulation of polyubiquitinated proteins and cell death.
1.5.3 Modulation of p97 activities

Since p97 is involved in such a wide variety of cellular processes, it is not surprising that normal activity of p97 is of crucial importance for cell survival. However, the molecular mechanisms that regulate activity of p97 in physiological and pathological conditions are still need to be identified. Similar to other AAA family members, p97 possesses weak ATPase activity, which is about 10-fold weaker than Na⁺/K⁺-ATPase (Song et al., 2003). In addition, the ATPase activity of p97 can be modulated by the level of ATP within a normal physiological concentration of ATP (0.5–2 mM), with the maximum at an ATP concentration of 3 mM (Song et al., 2003). The chaperone activity of AAA family proteins is often modulated by cellular environment. It has been shown that the ATPase activity of p97 is enhanced by high temperature and pH, but suppressed by salt, alcohol, ADP, and oxidizing agents (Song et al., 2003). Interestingly, p97 exhibits heat-enhanced ATPase activity, which peaks around 50–55 °C (Song et al., 2003). Since most enzymes are inactivated at such temperature, the stabilization of p97 may be attributed to its own chaperone activity.

In addition, a recent study identified that one cysteine residue (Cys522) is the critical site responsible for the regulation of p97 ATPase activity by oxidative stresses (Noguchi et al., 2005). Applying oxidative stresses (e.g., via low doses of hydrogen peroxide) resulted in a decreased ATPase activity of p97, which was due to oxidative modification of Cys522 (Noguchi et al., 2005). Replacement of Cys522 with the similar residue threonine, which cannot be oxidized, resulted in a p97 variant that resisted oxidative inactivation (Noguchi et al., 2005). In contrast, replacement of Cys522 with the residue lysine mimicking the oxidated state of cysteine resulted in decreased ATPase...
activity, aberrant ER expansion, induction of ER stress and cell death, which was highly similar to the expression of ATPase-deficient p97 (K524A) (Noguchi et al., 2005). Therefore, oxidative modulation of p97 is a crucial event that links oxidative stress and dysfunction of the ERAD pathway, triggering ER stress-mediated cell death (Noguchi et al., 2005).

Post-translational modifications, such as phosphorylation and acetylation, play important roles in regulation of p97 activity (Mori-Konya et al., 2009). p97 has been shown to be phosphorylated in at least six sites, namely Ser7, Ser352, Ser746, Ser748, Ser784 and Tyr805 (Egerton and Samelson, 1994; Klein et al., 2005; Lavoie et al., 2000; Rush et al., 2005; Vandermoere et al., 2006; Villen et al., 2007), although it has not been well studied whether these modifications affect the ATPase activities of p97. Tyrosine phosphorylation of Tyr805, which is localized in the C-terminal of p97, regulates transitional ER assembly \textit{in vitro}. Moreover, Lavoie et al. have identified membrane-associated JAK-2 and PTPH1 as the relevant kinase and phosphatase for Tyr805, respectively (Lavoie et al., 2000). They also demonstrated that Tyr805 dephosphorylation stabilizes the association between p97 and the ER membrane, hence promoting transitional ER assembly (Lavoie et al., 2000). Recently, p97 has been shown to be a target of Akt signaling in neuronal cells and in MCF-7 breast cancer cells (Klein et al., 2005; Vandermoere et al., 2007; Vandermoere et al., 2006). Akt is a serine/threonine kinase and an important mediator of cell survival and cell proliferation (Luo et al., 2003). p97 interacted and co-localized with Akt upon Akt activation (Klein et al., 2005; Vandermoere et al., 2006). Three serine residues, Ser352, Ser746 and Ser748, were identified to be phosphorylated by Akt upon stimulation (Klein et al., 2005;
Vandermoere et al., 2006). Replacement of these serines with alanines resulted in a p97 triple mutant (S352/746/748A) that markedly decreased the association of p97 with ubiquitinated proteins (Klein et al., 2005) and impaired the Akt-mediated pro-survival effect (Vandermoere et al., 2006).

1.5.4 p97 and NSF

p97 and NSF are distantly related members of the type II AAA ATPase family, and have comparable domain organization: an N domain of similar length followed by tandem copies of a specific ATP binding domain, known as the AAA domain. Amino acid sequencing showed significant homology (35%–50%) between NSF and p97 in regions near the ATP binding domains. Furthermore, both proteins have been implied in cellular morphology changes involving membrane compartments or vesicles.

Both p97 and NSF oligomerize into ring-shaped hexameric structures, which provide the mechanical basis for the chaperone activity. Like other AAA family members, NSF readily forms oligomers in the presence of nucleotides, but dissociate into monomers or dimers in the absence of nucleotides (Vale, 2000). In comparison, p97 is exceptionally stable in hexameric form, and hexamerization of p97 does not seem to require the presence of nucleotide (Wang et al., 2003a; Wang et al., 2003b), although it does accelerate hexamer formation. In NSF, the first (D1) domain is known to provide most, if not all, of the ATPase activity, while the second (D2) is primarily responsible for hexamerization (Nagiec et al., 1995; Whiteheart et al., 1994). In contrast, the reverse is true for p97: the D2 domain provides the bulk of the ATPase activity whereas the D1 domain appears to be responsible for hexamerization (Song et al., 2003; Wang et al., 2003a; Wang et al., 2003b). In addition, at a physiological temperature, p97 has a
significantly higher activity than NSF. These differences suggest that these two proteins may perform their relative tasks with different mechanisms of action.

The functional difference between p97 and NSF is obvious as NSF is mainly involved in the processes related to membrane fusion, whereas p97 mediates a wide variety of cellular processes (Brunger and DeLaBarre, 2003). The interaction of NSF with SNAREs requires the presence of adapter proteins, the soluble NSF attachment proteins (SNAPs) (Subramaniam et al., 1997). NSF and SNAP are ubiquitous components of NSF-driven membrane fusion (Barnard et al., 1997). In contrast, several distinct adapter proteins have been identified for p97: p47 (Ye et al., 2001), Ufd1/Npl4 (Meyer et al., 2000), VCIP135 (Uchiyama et al., 2002), and SVIP (small VCP interacting protein) (Nagahama et al., 2003). Except for VCIP135, all proteins interact with p97 in a mutually exclusive manner. This has led to a hypothesis that the various adapter proteins direct the ubiquitous p97 to different roles within the cell (Meyer et al., 2000).

Regardless of whether this hypothesis will be validated by further evidence (Meyer et al., 2002), no such variety of adapter proteins have been observed for NSF up to this point. Indeed, the other known target of NSF function, the GluR2 C-terminal tail (Jahn, 1998) appears to require the SNAP adapter protein as well (Hanley et al., 2002).

So, while this pair of AAA molecular machines could be considered as members of a class of proteins that work on membrane-bound proteins, the details of their structure, function, and mechanism make them very distinct from one another.
1.6 Working hypothesis and objectives

Activity-dependent long-term synaptic plasticity at hippocampal glutamatergic synapses, particularly LTP and LTD, has been proposed as the primary cellular substrate for learning and memory (Bliss and Collingridge, 1993; Kemp and Manahan-Vaughan, 2007; Martin et al., 2000). While a much better understanding of the molecular mechanisms mediating both LTP and LTD exists today than a few years ago, we still know very little about the particular roles of these forms of plasticity in learning and memory. This is due in large part to a lack of specific inhibitors for either LTP or LTD. Previous studies of the long-term spatial memory in the Morris water maze found that it can be abolished by preventing NMDAR activation using either pharmacological or genetic approaches (Bliss and Collingridge, 1993; Morris et al., 1986; Sakimura et al., 1995; Tsien et al., 1996). However, due to a similar requirement of NMDAR activation by these two forms of plasticity (Collingridge et al., 1983; Dudek and Bear, 1992), the particular roles of LTP and LTD in long-term spatial memory still need to be determined. Given the different molecular mechanisms of LTP and LTD, such as interacting proteins and signaling pathways, we hypothesize that 1) Hippocampal LTP and LTD can be selectively blocked by antagonists against NMDARs carrying different NR2 subunits in freely moving rats; 2) The bidirectional synaptic plasticities in hippocampus play distinct roles in the formation of long-term spatial memory (LSM) by applying the specific LTP or LTD inhibitors in a well-studied behavior task, the Morris water maze; 3) A more specific LTP inhibitor will be developed by studing AMPAR interacting protein partners and mechanisms underlying these protein-protein interactions. The main objectives include the followings:
1. Determining whether the pharmacological approaches that abolish LTP or LTD in brain slices can be used in freely-moving rats. Evidence accumulated from recent studies suggests that NR2A- and NR2B-containing NMDARs, respectively, contribute to the induction of hippocampal LTP and LTD in both *in vitro* (brain slices) and *in vivo* (anesthetized animals) (Duffy et al., 2008; Fox et al., 2006; Kohr et al., 2003; Liu et al., 2004a; Woo et al., 2005) (but see also (Berberich et al., 2005; Morishita et al., 2007)). Since the NR2A (NVP-AAM077(Auberson et al., 2002)) and NR2B antagonists (Ro25-6981) used in these studies cross the blood brain barrier (Fox et al., 2006), they should be effective in acutely inhibiting LTP and LTD formation in behaving rats. Moreover, Tat-GluR23Y peptide, which inhibits clathrin-dependent endocytosis of AMPARs (Ahmadian et al., 2004; Brebner et al., 2005; Lin et al., 2000; Man et al., 2000), has been used as a specific inhibitor for LTD in many studies (Brebner et al., 2005; Kim et al., 2007; Wong et al., 2007). To confirm their specificity in freely moving rats, we examined the effects of these specific NR2 antagonists and Tat-GluR23Y peptide on bidirectional hippocampal synaptic plasticity in freely moving rats with *in vivo* electrophysiological recordings.

2. Determining whether inhibiting LTP or LTD affect the long-term spatial memory. Once the specificity of the drugs in blocking LTP or LTD in freely-moving rats were identified, we subsequently examined the impacts of blocking hippocampal LTP or LTD on LSM formation in MWM. Furthermore, in order to confirm whether the influence on LSM by these drugs is due to inhibiting LTP or LTD per se, a visible MWM task was performed to test if the visual or sensorimotor functions are normal after drug application.

3. To look for a specific LTP inhibitor that targets AMPAR trafficking. Due to the lack of specific peptide-based LTP inhibitor that targets the facilitated exocytosis of AMPARs
(the last step in the expression of LTP), the distinct role of LTP in the long-term spatial memory cannot be clearly identified as LTD, which can be blocked using a Tat-GluR2_{3Y} peptide. As the expression of LTP is thought to be mediated by a facilitation of exocytosis of AMPARs into the postsynaptic membrane via a mechanism involving GluR1 and its interacting protein partners, we hypothesize that further characterizing detailed partners and mechanisms underlying these protein-protein interactions may lead to the development of a much more specific LTP inhibitor. Thus, specific efforts were made to identify novel GluR1 specific protein binding partner(s) using co-immunoprecipitation with anti-GluR1 or anti-GluR2 antibody followed by mass spectrometric analysis. Afterwards, the functions of these proteins on AMPAR trafficking and synaptic plasticity were examined with the aim to identify a new specific peptide-based inhibitor for LTP. Thus, the role of LTP in the LSM formation was tested using this LTP inhibitor.

By completion of this work, I expect to establish the notion that bidirectional hippocampal synaptic plasticities play distinct roles in the long-term spatial memory. Moreover, the study of novel AMPAR associated proteins will enrich our understandings of the molecular mechanisms of AMPAR trafficking and its roles in the synaptic plasticity and long-term spatial memory.
1.7 References


Perez, J.L., Khatri, L., Chang, C., Srivastava, S., Osten, P., and Ziff, E.B. (2001). PICK1 targets activated protein kinase Calpha to AMPA receptor clusters in spines of hippocampal neurons and reduces surface levels of the AMPA-type glutamate receptor subunit 2. J Neurosci 21, 5417-5428.


receptors for hippocampal synaptic plasticity but not for spatial learning. Science 284, 1805-1811.


2. HIPPOCAMPAL LONG-TERM DEPRESSION IS REQUIRED FOR THE CONSOLIDATION OF LONG-TERM SPATIAL MEMORY

2.1 Introduction

The critical roles of the hippocampus in encoding and consolidating certain forms of memory have been well documented in several mammalian species including humans (Morris et al., 1982; Scoville and Milner, 1957; Squire, 1992). Activity-dependent long-term synaptic plasticity at hippocampal glutamatergic synapses, particularly NMDAR-dependent LTP and LTD, has been proposed as the primary cellular substrate for fulfilling these cognitive roles (Bliss and Collingridge, 1993; Kemp and Manahan-Vaughan, 2007; Martin et al., 2000). For instance, formation of long-term spatial memory (LSM) in the Morris water maze (MWM) can be abolished by preventing NMDAR activation using either pharmacological or genetic approaches (Bliss and Collingridge, 1993; Morris et al., 1986; Sakimura et al., 1995; Tsien et al., 1996). Since LTP and LTD alter glutamatergic transmission in opposing ways, they likely play distinct roles in LSM formation. However, due to the similarities shared by these two forms of plasticity, such as the requirement of NMDAR activation (Collingridge et al., 1983; Dudek and Bear, 1992), differentiating the functional roles of LTP and LTD has been technically difficult. Recent attempts using genetic approaches to generate transgenic mice with deficits in either LTP (Abel et al., 1997; Kohr et al., 2003; Zamanillo et al., 1999) or LTD (Nicholls et al., 2008; Zeng et al., 2001) have achieved some success in delineating the contribution of these two opposing forms of plasticity in LSM. However,

---

1 A version of this chapter will be submitted for publication. Ge, Y., Dong, Z., Bagot, R.C., Howland, J.G., Phillips, A.G., Wong, T.P., and Wang, Y.T. Hippocampal long-term depression is required for the consolidation of long-term spatial memory.
results obtained from these mice were hardly equivocal, with some transgenic mice exhibiting intact LSM (Bannerman et al., 2008; Nicholls et al., 2008; Reisel et al., 2002; Zamanillo et al., 1999; Zeng et al., 2001). Although findings obtained from LTP- and LTD-null animals raised doubts as to the requirement of hippocampal synaptic plasticity in LSM formation, the integrity of LSM in these animals could be related to compensatory changes arising after prolonged genetic alterations (throughout brain development or for weeks in inducible knock-down studies). Developing experimental approaches that acutely inhibit LTP or LTD in genetically unaltered animals may overcome some of the limitations associated with these transgenic studies and thereby provide a better understanding of the functional contributions of hippocampal LTP and LTD to LSM formation.

Evidence accumulated from recent studies suggests that NR2A- and NR2B-containing NMDARs, respectively, contribute to the induction of hippocampal LTP and LTD in both in vitro in brain slices and in vivo in anesthetized animals (Duffy et al., 2008; Fox et al., 2006; Kohr et al., 2003; Liu et al., 2004a; Woo et al., 2005) (but see also (Berberich et al., 2005; Morishita et al., 2007)). Since the NR2A (NVP-AAM077 (NVP (Auberson et al., 2002))) and NR2B antagonists (Ro25-6981 (Ro)) used in these studies cross the blood brain barrier (Fox et al., 2006), they should be effective in acutely inhibiting LTP and LTD formation in behaving rats. To confirm this, we examined the effects of these specific NR2 antagonists on bidirectional hippocampal synaptic plasticity in freely moving rats. Subsequently, we examined the impact of blocking hippocampal LTP and LTD on LSM formation in MWM. In addition to demonstrating that hippocampal LTP and LTD in freely moving rats can be prevented by NR2A antagonist
NVP and NR2B antagonist Ro, respectively, surprisingly, we also found that LSM formation in MWM remained largely intact despite blocking LTP formation by NVP. In contrast, the consolidation of LSM was totally abolished when LTD formation was blocked by Ro. Notably, LSM consolidation was similarly impaired by Tat-GluR23Y peptide, which inhibits clathrin-dependent endocytosis of AMPARs (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid subtype of glutamate receptor), a mechanism of LTD expression (Ahmadian et al., 2004; Brebner et al., 2005; Lin et al., 2000; Man et al., 2000). Taken together, our findings revealed that acute blockade of hippocampal LTD, but not LTP, is sufficient to abolish the consolidation of LSM.
2.2 Methods

2.2.1 Animals

All experiments were performed on male Sprague-Dawley rats (300 to 400g). The rats were housed with cage-mates in cages with free access to food and water, and maintained at 21 ± 2 °C with a 12 h light-dark cycle. All experiments were performed in accordance with the Canadian Council on Animal Care and were approved by the University of British Columbia and Douglas Institute Animal Care Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2.2 Morris water maze (MWM)

Long-term spatial memory (LSM) was assessed using a MWM task as previously described (Moghaddam, 1993). Briefly, the test was divided into the training phase on day 1 and the retrieval phase 24 h later. The maze apparatus consisted of a 2-m diameter fiberglass pool filled with 27-29 °C water. A square platform (15 cm × 15 cm) in a fixed location was either submerged 2 cm below the water (for hidden platform version of MWM) or 2 cm above the water surface (for visible platform MWM). The water was made opaque by the addition of nontoxic black paint. The pool was surrounded by light blue curtains to which three large cues made from white corrugated plastic were attached. The room was dimly lit with four desk lamps pointed at the ceiling in each corner of the room. Care was taken to ensure that no shadows were cast in the pool. During the training phase, each rat was trained over eight consecutive trials to find the platform. The starting point of each trial was selected from the four compass points of the pool in an identical but randomized sequence (N, S, E, W, S, N, W, E). Rats were allowed to swim
freely to find the platform. If rats failed to find the platform within 60 s, they were gently guided to it by the experimenter. Rats remained on the platform for 20 s before being removed by the experimenter. Between each training trial, rats were placed in a plastic 'shoebox' cage for 30 s. Probe tests, during which the platform was removed from the pool, were performed 24 hrs or 30 s after training. Briefly, rats were placed in the pool from a novel drop point (between the N and E compass points) and allowed to swim for 60 s. All trials were recorded with a video camera and analyzed using an EthoVision tracking system (Noldus, Leesburg, VA).

2.2.3 Bilateral hippocampal microinjection

Surgery: Rats were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.). Atropine (0.1 mg/kg, i.p.) was also given to help relieve respiratory congestion. Scalp fur was shaved by a clipper and disinfected with iodine before rats were mounted on a stereotax. After opening the scalp skin and exposing the skull, two 22-gauge stainless steel guide cannulae (10 mm; Small Parts Inc., Miami Lakes, FL) were implanted above the dorsal hippocampus (3.8 mm posterior to bregma, 2.5 mm lateral to the midline and 2.5 mm below the surface of the dura) and were fixed to the skull with four jeweler’s screws and dental cement. Sterile stainless steel stylets (30 Ga metal rod, ~8 mm in length, Small Parts Inc., Miami Lakes, FL) were inserted into guide cannulae to avoid bacterial infection and aCSF leakage through the cannulae. All rats were allowed to recover for at least 10 days before habituation. Habituation: Before drug injection experiments, rats were extensively habituated to the injection procedures by having their stylets removed and being placed into one of the two Plexiglas injection boxes (32 × 32 × 41 cm) with dummy needles inserted into their guide cannulae. Dummy needles (2 mm
Habituation sessions were conducted 3-4 times for each rat in the week preceding the water maze training. Intra-hippocampal drug injection: Two stainless steel needles (11 mm) were inserted into the dorsal hippocampi extending 1 mm beyond the tip of the guide cannulae. Drugs were injected with 25-µl Hamilton syringes and a microsyringe pump (Harvard Apparatus) at 0.5 µl/min for 2 min. The needles were left in place for an additional minute to allow the diffusion of the drug away from the needle tips. The rats were then removed from the injection boxes, their stylets replaced, and returned to their home cages for 15 mins. Rats were then either transported back to the housing facility (post-training injection) or trained in the water maze (pre-training injection).

2.2.4 Histology

Rats were overdosed with sodium pentobarbital, perfused with 0.1 M PBS, and their brains were placed in 10% formalin/10% sucrose for a minimum of 1 week. To determine the site of injection, brains were cut into 60-µm sections on a cryostat and stained with cresyl violet. Sections containing the cannulae tracts were examined under a light microscope and the location of cannulae were recorded on the appropriate plates of a rat brain atlas (Paxinos, 1986).

2.2.5 Recording of hippocampal synaptic plasticity in freely moving rats

Surgery and electrode implantation: Rats were chronically implanted with electrodes as previously described (Dong et al., 2008; Manahan-Vaughan and Braunewell, 1999; Xu et al., 1998). Briefly, rats were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and pretreated with atropine to prevent excessive salivation. The core
temperature of anesthetized rat was maintained at 36.5 ± 0.5°C. Three stainless-steel screws (1.59 mm diameter) were inserted into the skull through drill holes without piercing the dura. One served as a ground electrode (7 mm posterior to bregma and 5 mm left of the midline), another acted as an anchor (opposite the ground screw, 7 mm posterior to bregma and 5 mm right of the midline) and the third served as the reference electrode (8 mm anterior to bregma and 1 mm left of the midline). Recording and stimulating electrodes were constructed by gluing together a pair of twisted Teflon-coated 90% platinum/10% iridium wires (50 μm inner diameter, 75 μm outer diameter, A-M Systems Inc., USA). The recording electrode was lowered into the CA1 region (3.8 mm posterior to bregma, 2.8 mm right of the midline and 2.5 mm below the surface of the dura), and the stimulating electrode was placed in the Schaffer collaterals of the dorsal hippocampus (4.5 mm posterior to bregma, 3.8 mm right of the midline and 2.8 mm below the dura) via holes drilled through the skull. The electrode socket assembly was fixed onto the skull with dental cement. The correct placement of the electrodes in the CA1 region of the dorsal hippocampus was verified by electrophysiological criteria (Leung, 1979) and histological examination (see Histology). Electrophysiology: All rats were allowed to recover for at least 10 days before in vivo recording. During this recovery period, rats were habituated to the recording chamber (40 × 40 × 60 cm), which was made of black Plexiglas walls and open at the top, for at least 1 h each day. To allow rats to move around freely in the chamber during recording, implanted electrodes were connected by a flexible cable and swivel commutator (Crist Instrument Co., Inc., USA) to the stimulation and recording equipment. Field excitatory postsynaptic potentials (fEPSPs) were evoked by using square-wave stimulations (pulse width = 0.12 ms). Test
fEPSPs were evoked at a frequency of 0.033 Hz and at a stimulus intensity adjusted to around 50% of the maximal response size. fEPSPs evoked from six consecutive stimulations over 3 mins were averaged to obtain a data point. The first ten data points (30 mins) served as baseline. In the present experiment, two low-frequency stimulation (LFS) protocols were used to elicit LTD: 1) classical LFS (900 pulses at 1 Hz); 2) paired-burst stimulation (PB, 200 pairs of two-pulse bursts at one pair per second, with an interval of 2.5 ms between pulses and 10 ms between bursts). To elicit LTP, high-frequency stimulation (HFS) was given, consisting of 100 pulses at 100 Hz.

2.2.6 Statistical analysis

Water maze: All data are presented as mean ± SEM. The time spent in the test and opposite quadrants during the probe test in each experiment (NVP, Ro, and Tat-GluR23Y peptide) was analyzed by ANOVA. Post hoc analyses were conducted with Fisher’s test for between-subjects comparisons and paired Student’s t tests for within-subjects comparisons.

Electrophysiological experiments: All data are expressed as the average percent change from baseline ± SEM and were analyzed by one-way ANOVA followed by post hoc Fisher’s tests where appropriate.
2.3 Results

2.3.1 Impact of NR2A and NR2B antagonists on bidirectional hippocampal synaptic plasticity recorded from freely moving adult rats

We first established an *in vivo* model of hippocampal synaptic plasticity in freely moving adult rats. Stimulating and recording electrodes were implanted in anesthetized male SD rats as previously described (Dong et al., 2008; Manahan-Vaughan and Braunewell, 1999; Xu et al., 1998). After a > 10-day recovery period, Schaffer-Collateral stimulation-induced *field excitatory postsynaptic potentials* (fEPSP) were recorded from the hippocampal CA1 region in freely moving rats under non-anesthetized conditions. We found that LTP could be reliably induced by *high frequency stimulation* (HFS, 100 Hz, 100 pulses; saline-treated rats: fEPSP slope at 55-60 mins (same time points were used in subsequent analyses) after HFS was 144.3 ± 5.3 % of pre-induction; n = 8, p = 0.001 vs. baseline, Fig. 2.1A). A standard *low-frequency stimulation* protocol (LFS, 1 Hz, 900 pulses) failed to trigger LTD (saline-treated rats: 102.5 ± 4.8 %, n = 6, p = 0.368 vs. baseline, data not shown). This finding is in a good agreement with previously reported difficulties in inducing hippocampal LTD in adult rats (Bear and Abraham, 1996; Kemp et al., 2000). However, a robust LTD (saline-treated rats: 78.5 ± 2.8 %, n = 6, p = 0.001 vs. baseline, Fig. 2.1B) could be induced using a stronger *paired-burst stimulation protocol* (PB, 200 pairs of two-pulse bursts at one pair per second, with an interval of 2.5 ms between pulses and 10 ms between bursts). The *in vivo* LTP and LTD recorded in freely moving rats were NMDAR-dependent and could be prevented by administering a subunit-nonspecific NMDAR antagonist CPP (10 mg/kg, i.p.) 30 mins prior to induction (CPP-treated rats, 103.0 ± 5.8 % after HFS, n = 5, p = 0.302 vs. baseline, data not shown).
Figure 2.1 NR2A and NR2B antagonists prevent the formation of hippocampal LTP and LTD, respectively, in freely moving adult rats

(A) High frequency stimulation (HFS; 100 Hz, 100 pulses) triggered LTP of fEPSP recorded from the hippocampal CA1 region of freely moving rats. The scatter plots show the effect of pre-training (30 mins before training) i.p. injection of vehicle (saline), a subunit non-selective NMDAR antagonist CPP (10 mg/kg), a NR2A antagonist NVP (1.2 mg/kg), and a NR2B antagonist Ro (6 mg/kg) on in vivo hippocampal LTP formation in freely moving adult rats. Note that HFS failed to induce LTP in CPP- and NVP- treated rats. Histograms in the middle summarize the percentage change in field excitatory postsynaptic potential slope (fEPSP slope) 5 mins before (x) and 55 mins after HFS (y). Representative traces of fEPSP recorded from each treatment group at these time points are shown on the right. (B) Paired-burst stimulation (PB, 200 paired-burst at 1 Hz; inter-pulse interval = 2.5 ms; inter-burst interval = 10 ms) induced LTD of fEPSP recorded from the hippocampal CA1 region of freely moving rats. The effects of different drug treatments on in vivo LTD in freely moving rats are summarized in both the scatter plot (left) and histogram (middle). LTD was induced by PB in only control and NVP-treated rats. Representative traces of fEPSP recorded from each treatment group are shown on the right. * p < 0.05 vs. control (saline-treated group), post-hoc Fisher’s test after ANOVA.
baseline, p = 0.001 vs. saline-treated rats, Fig. 2.1A; 97.5 ± 5.8 % after PB; n = 4; p = 0.355 vs. baseline; p = 0.011 vs. saline-treated rats; Fig. 2.1B).

We next determined if NR2A- and NR2B-containing NMDARs are necessary for LTP and LTD formation in freely moving rats. As shown in Fig. 2.1A, we found that intraperitoneal (i.p.) injection of NVP (1.2 mg/kg) 30 mins before HFS prevented LTP formation (101.6 ± 2.6 %, n = 7, p = 0.121 vs. baseline, p = 0.017 vs. saline-treated rats). The same dose of NVP has been shown to prevent in vivo LTP formation in anesthetized rats (Fox et al., 2006). However, NVP did not affect PB-induced LTD (79.5 ± 3.1 %, n = 5, p = 0.002 vs. baseline, p = 0.997 vs. saline-treated rats; Fig. 2.1B). Similar to our previous findings obtained from anesthetized rats (Fischer et al., 1997; Wong et al., 2007), Ro (6 mg/kg, i.p.) failed to affect LTP formation (142.5 ± 1.3 %; n = 4; p = 0.001 vs. baseline; p = 0.415 vs. saline-treated rats; Fig. 2.1A) but prevented PB-induced LTD (96.5 ± 2.7 %; n = 7; p = 0.081 vs. baseline; p = 0.001 vs. saline-treated rats; Fig. 2.1B). Thus, these results strongly indicate that the induction of hippocampal LTP and LTD in freely moving adult rats was prevented by systemic injection of NR2A antagonist NVP and NR2B antagonist Ro, respectively.

2.3.2 Effect of blockade of hippocampal LTP or LTD by NR2 antagonists on LSM formation

Having confirmed the specificity of NVP and Ro in preventing hippocampal LTP and LTD formation in behaving rats, we then used these antagonists to differentiate the contribution of bidirectional hippocampal synaptic plasticity to LSM formation in a single training day MWM protocol (de Quervain et al., 1998; Wong et al., 2007). Briefly,
rats were trained to remember the location of a hidden platform over eight trials within 30 mins (learning, Fig. 2.2B). Following a twenty-four hour consolidation period (memory consolidation), a 60s probe test with the platform removed was performed (memory retrieval, Fig. 2.2C). LSM of the hidden platform location has been shown to be reliably induced by this one day training protocol (de Quervain et al., 1998; Frick et al., 2004; Wong et al., 2007). In comparison with other commonly used versions of MWM with multiple training days, this protocol has the clear advantage of reducing the overlap between the learning and memory consolidation phases so that the contribution of synaptic plasticity to these specific phases of memory processing can be better differentiated. The effects of preventing LTP or LTD on learning were investigated by i.p. injection of either vehicle or one of the NR2 antagonists 30 mins before training.

We found that neither NVP nor Ro affected the acquisition of learning the location of a hidden platform across 8 training trials. The averaged escape latencies in the first 4 trials were significantly longer than those in the last four trials in all treatment groups (saline, NVP, Ro), and no effect of drug treatment was observed (first 4 trials: $F(2,34) = 2.13, p = 0.14$; in the last 4 trials: $F(2,34) = 2.05, p = 0.15$, Fig. 2.2B). In the retrieval phase, we found that saline-treated rats spent significantly longer in the test quadrant ($Q_{\text{test}}$, where the hidden platform was formerly placed; $19.7 \pm 1.8$ s, $n = 11$, Fig. 2.2C) than in the opposite quadrant ($Q_{\text{opp}}$, $10.6 \pm 1.2$ s, $n = 11$, $p = 0.009$ for $Q_{\text{test}}$ vs. $Q_{\text{opp}}$). Similar preference for $Q_{\text{test}}$ was observed in the comparison of swimming distance ($Q_{\text{test}}$ $6.20 \pm 0.39$ m vs. $Q_{\text{opp}}$ $3.48 \pm 0.38$ m, $n = 11$, $p = 0.001$, data not shown). Surprisingly, preventing LTP formation by NVP did not affect the preference for $Q_{\text{test}}$ ($Q_{\text{test}}$ $16.9 \pm 1.0$ s vs. $Q_{\text{opp}}$ $12.1 \pm 1.3$ s, $n = 13$, $p = 0.019$, Fig. 2.2C). In contrast, we found that the $Q_{\text{test}}$
Figure 2.2 LSM formation is affected if the induction of hippocampal LTD is prevented during MWM training

(A) On day 1, rats received i.p. injection of drugs 30 mins before 8 training trials. Probe test without the hidden platform was performed on day 2. (B) Histograms display the averaged escape latencies of rats that received different treatments during the first 4 (trials 1-4) and last 4 training trials (trials 5-8) on day 1. (C) Another set of histograms reveal probe test performance of rats from different treatment groups on day 2. LSM of the hidden platform location is indicated by preference for the test quadrant ($Q_{\text{test}}$), where the hidden platform was formerly placed, over the opposite quadrant ($Q_{\text{opp}}$). The schematic diagram shows the location of these two quadrants and the hidden platform. Dotted lines indicate chance performance (15s). (D and E) Histograms in these panels summarize the effect of Ro and NVP on other probe test performances such as the number of hidden platform crossings and the latency of first platform crossing (D), and thigmotactic behaviour defined as swimming in the perimeter (outer ring) of the pool (E). * $p < 0.05$ between the first 4 and the last 4 trials, paired Student’s t-test in (B); * $p < 0.05$ between time in $Q_{\text{test}}$ and time in $Q_{\text{opp}}$, paired Student’s t-test in (C); # $p < 0.05$ vs. saline treated control, post-hoc Fisher’s test after ANOVA (D, E).
preference was abolished in Ro-treated rats, which spent similar amounts of time swimming in both test and opposite quadrants ($Q_{test} 15.1 \pm 1.5 \text{ s}$ vs. $Q_{opp} 13.4 \pm 1.2 \text{ s}$, $n = 13$, $p = 0.516$, Fig. 2.2C).

In addition to impairing the $Q_{test}$ preference, Ro also affected other probe test performance indices. We found that Ro-treated rats crossed the hidden platform location fewer times than other groups (saline, $1.55 \pm 0.39 \text{ n = 11}$; NVP, $1.23 \pm 0.34 \text{ n = 13}$; Ro, $0.46 \pm 0.18 \text{ n = 13}$; $F(2,34) = 3.22$; post-hoc: saline vs. NVP, $p = 0.485$; saline vs. Ro, $p = 0.020$, Fig. 2.2D). In addition, Ro-treated rats exhibited longer latencies to cross the hidden platform location (saline, $26.4 \pm 6.2 \text{ s}$, $n = 11$; NVP, $30.6 \pm 6.6 \text{ s}$, $n = 13$; Ro, $48.8 \pm 5.0 \text{ s}$, $n = 13$; $F(2,34) = 4.02$; post-hoc: saline vs. NVP, $p = 0.631$; saline vs. Ro, $p = 0.013$, Fig. 2.2D). Since we trained rats with a weak training protocol of only 8 trials, we observed some rats in all drug groups that did not pass over the hidden platform during probe tests at all. Notably, the percentage of animals that did not pass the hidden platform location during the 60s probe test in the Ro-treated group (62%, 8 out of 13) was higher than that in the saline- (18%, 2 out of 11) and NVP-treated groups (31%, 4 out of 13). We next investigated the thigmotactic behavior of rats (swimming close to the perimeter of the pool) by dividing the pool into three equally spaced concentric circles (radius of 0.33, 0.66, and 1 m). We found that Ro-treated rats spent significantly longer than other rats in the outer ring (saline, $32.4 \pm 2.0 \text{ s}$, $n = 11$; NVP, $31.8 \pm 2.1 \text{ s}$, $n = 13$; Ro, $40.9 \pm 1.6 \text{ s}$, $n = 13$; $F(2,34) = 7.22$; post-hoc: saline vs. NVP, $p = 0.808$; saline vs. Ro, $p = 0.004$, Fig. 2.2E). Remarkably, this thigmotactic behavior was similar to that observed in naive saline-treated rats during the first training trial ($43.0 \pm 3.2 \text{ s}$, $n = 11$, Fig. 2.2E). The compromised probe test performances of Ro-treated rats were not caused a
deficit of motor performance, since neither NMDAR antagonist affected swimming speed (saline, 0.329 ± 0.012 ms⁻¹, n = 11; NVP, 0.329 ± 0.010 ms⁻¹, n = 13; Ro, 0.336 ± 0.011 ms⁻¹, n=13; F(2,34) = 0.16; post-hoc: saline vs. NVP, p = 0.998; saline vs. Ro, p = 0.644).

In summary, our findings strongly suggest that LSM formation is highly sensitive to blockade of NR2B containing NMDARs by pre-training injection of the NR2B antagonist Ro.

Previous studies have shown that an NMDAR antagonist could inhibit long-term memory retrieval in a state-dependent manner: i.e. the presence of NMDAR antagonists in the brain during learning created a chemical state so that the reinstatement of the same state is required for memory retrieval (Jackson et al., 1992; Overton, 1985). If the LSM impairment caused by pre-training injection of Ro is related to state-dependent learning, we expected that the presence of Ro during probe tests would rescue LSM retrieval. However, the disruption of Qtest preference caused by pre-training Ro injection was not different between rats that received Ro or saline 30 mins before the probe test (Fig. 2.3). State-dependent learning is therefore unlikely to account for the impact of Ro on LSM formation. Instead, preventing hippocampal LTD, the cellular process that is selectively affected by Ro, is the probable explanation for the deficit of LSM formation in Ro-treated rats.

2.3.3 Effect of preventing the expression of hippocampal LTP or LTD by Tat-GluR23y peptide on LSM formation

If hippocampal LTD is a crucial plastic process for LSM formation, blocking this plastic change via an alternative non-NMDAR targeting approach should produce a
Figure 2.3 Impact of pre-training injection of Ro on LSM formation was not caused by state-dependent learning

(A) 30 mins before training (Drug A) and probe test (Drug B), rats were injected with either Ro or saline in combinations shown in (B). (B) Histograms show the probe test performance of rats that received different combinations of drugs. Note that preference for $Q_{test}$ was disrupted in rats that received pre-training injection of Ro. Importantly, pre-probe test injection of Ro failed to rescue this deficit. * $p < 0.05$ between time in $Q_{test}$ and time in $Q_{opp}$, paired Student’s t-test.
similar behavioral deficit. Recently, it has been shown that LTD expression is related to the clathrin-dependent endocytosis of AMPARs (Lin et al., 2000; Man et al., 2000). AMPAR endocytosis is regulated by the phosphorylation status of AMPAR subunits, in particular the GluR2 subunit (Chung et al., 2000; Hayashi and Huganir, 2004). We have found that phosphorylation of the three tyrosine residues on the carboxyl tail of the GluR2 subunit is crucial for AMPAR endocytosis (Ahmadian et al., 2004). Indeed, interfering with the phosphorylation of these residues on endogenous AMPARs by a short GluR23Y peptide that contains these tyrosine residues (YKEGYNVYG) abolished LTD formation in both the hippocampus (Ahmadian et al., 2004) and nucleus accumbens (Brebner et al., 2005). We examined the impact of preventing LTD expression on LSM formation using a version of this peptide rendered membrane and blood brain barrier permeable by conjugation to a membrane transduction Tat domain (YGRKKRRQRRR) from HIV virus (Schwarze et al., 1999). This Tat-GluR23Y peptide has been systemically administered by us (Brebner et al., 2005; Wong et al., 2007) and others (Van den Oever et al., 2008) into freely moving rats to examine the functional roles of LTD in different behaviors.

We first investigated if Tat-GluR23Y peptide selectively inhibits hippocampal LTD formation in freely moving rats. Systemic injection (3 µmol/kg, i.p.) of Tat-GluR23Y peptide, but not the scrambled peptide control (Tat-VYKYGGYNE), 30 mins before PB stimulation prevented hippocampal LTD formation in freely moving rats (Tat-GluR23Y peptide-treated rats: 98.3 ± 5.8 %, n = 6, p = 0.413 vs. baseline, p = 0.017 vs. scrambled peptide-treated rats at 79.1 ± 3.3 %, n = 5, p = 0.001 vs. baseline, Fig. 2.4A). However, Tat-GluR23Y peptide treatment did not affect LTP induced by HFS (Tat-
Figure 2.4 LSM formation is affected if the expression of hippocampal LTD is inhibited during MWM training

(A) Scatter plots show the effect of pre-training (30 mins before training) i.p. injection of active (Tat-GluR23Y peptide), and control peptide (scrambled Tat-GluR23Y peptide; 3 μmol/kg) on in vivo LTD induced by paired-burst stimulation (PB; left) and on in vivo LTP induced by high frequency stimulation (HFS; right) in freely moving rats. Note that only PB-induced LTD was affected by the active Tat-GluR23Y peptide. (B) Histograms display the averaged escape latencies during the first 4 (trial 1-4) and last 4 training trials (trial 5-8) from rats that received systemic injection (i.p.) of either vehicle (saline) or one of the Tat-peptides 30 mins before training (day 1). (C) Another set of histograms reveal probe test performance (day 2) of rats that received pre-training systemic injection of drugs. Dotted lines represent chance (15s). The preference for Q_{test} in MWM-trained rats was abolished in Tat-GluR23Y peptide-treated rats. (D) Histograms show the effects of pre-training intra-hippocampal injection (15 mins before probe test) of vehicle and peptides on probe test performance 24 hrs after training. Direct injection of peptide before training disrupted the Q_{test} preference of MWM-trained rats in probe tests. (E) The impact of intra-hippocampal injection of saline or Tat-GluR23Y peptide on performance in a visible platform version of MWM is represented in a histogram. Briefly, 15 mins after intra-hippocampal drug injection, rats were trained to find a visible platform (2 cm above water) in eight 60 s training trials. The histogram shows that pre-training intra-hippocampal injection of Tat-GluR23Y peptide did not affect the latency to locate the visible platform 24 hrs after training. (F) Nissl-stained coronal section of rat brain showed cannulae placement above the dorsal hippocampus. * p < 0.05 between the
first 4 and the last 4 trials, paired Student’s t-test in (B); * $p < 0.05$ between time in $Q_{\text{test}}$ and time in $Q_{\text{opp}}$, paired Student’s t-test in (C) and (D).
GluR2$_{3Y}$ peptide-treated rats at 55-60 mins after HFS: 130.1 ± 9.1 %, n = 6, p = 0.012 vs. baseline, p = 0.251 vs. scrambled peptide-treated rats at 139.51 ± 5.9 %, n = 5, p = 0.001 vs. baseline, Fig. 2.4A), suggesting that Tat-GluR2$_{3Y}$ peptide specifically blocks LTD formation in freely moving rats.

Next, we examined the impact of Tat-GluR2$_{3Y}$ peptide on LSM formation in MWM. We found that rats treated with both the active and control peptides showed similar acquisition of the hidden platform location (first 4 trials: $F(2,20) = 0.86$, $p = 0.44$; in the last 4 trials: $F(2,20) = 1.23$, $p = 0.32$, Fig. 2.4B). However, systemic administration of the peptide (30 mins before MWM training) disrupted rats’ preference for $Q_{\text{test}}$ in probe tests performed 24 hrs after training ($Q_{\text{test}} 16.2 ± 1.4$ s vs. $Q_{\text{opp}} 13.0 ± 1.5$ s; n = 14, $p = 0.259$, Fig. 2.4C). The effect of the peptide was not related to the biologically active Tat domain because using a control scrambled peptide, which contains an intact Tat domain and a scrambled GluR2$_{3Y}$ sequence, failed to produce the same behavioral impairment ($Q_{\text{test}} 18.7 ± 1.4$ s vs. $Q_{\text{opp}} 11.8 ± 1.5$ s; n = 8, $p = 0.031$, Fig. 2.4C). Similar to the effect of Ro, we found that pre-training injection of Tat-GluR2$_{3Y}$ peptide was sufficient to impair LSM formation.

Systemic injection of a peptide results in diffusion throughout the brain and may affect central processes in brain regions other than the hippocampus. To confirm that LTD in the hippocampus in particular is required for LSM formation, we examined probe test performances of rats that received bilateral intra-hippocampal injection of Tat-GluR2$_{3Y}$ or scrambled peptides 15 mins before MWM training. We found that while rats that received bilateral intra-hippocampal injection of vehicle (PBS, 1 µl) displayed normal preference for the test quadrant in probe tests ($Q_{\text{test}} 21.2 ± 2.6$ s vs. $Q_{\text{opp}} 7.8 ± 2.4$
s; n = 6, p = 0.042, Fig. 2.4D), rats that received intra-hippocampal injection of Tat-GluR23Y peptide (30 pmol, 1 µl) failed to show the same preference (Qtest 15.5 ± 0.6 s vs. Qopp 12.3 ± 1.2 s; n = 6, p = 0.078, Fig. 2.4D). However, intra-hippocampal injection of a scrambled peptide did not affect rats’ probe test performance (Qtest 17.9 ± 1.2 s vs. Qopp 11.8 ± 0.6 s; n = 6, p = 0.016, Fig. 2.4D). Importantly, direct intra-hippocampal injection of Tat-GluR23Y peptide did not affect rats’ performances in a visible platform task (Fig. 2.4E), which suggests that any tissue damage due to the drug injection procedure did not affect rat’s ability to perform the non-spatial requirements of the MWM. In parallel to our findings obtained from systemic injection of Ro and Tat-GluR23Y peptide, we found that preventing the formation of hippocampal LTD by intra-hippocampal infusion of Tat-GluR23Y peptide impaired LSM formation in MWM. Since both Ro and Tat-GluR23Y peptide affected LSM in a similar manner, our findings strongly support the conclusion that a common central process that is targeted by these two chemically distinct drugs, hippocampal LTD, is required for LSM formation.

2.3.4 LTD inhibitors do not affect the acquisition of spatial memory

Spatial memory formation can be arbitrarily divided into acquisition, consolidation, and retrieval phases. Since we found that hippocampal LTD but not LTP is likely required for LSM formation, we investigated the functional contribution of LTD at different stages of LSM formation. Although pre-training administration of LTD inhibitors (Ro or Tat-GluR23Y peptide) affected rats’ performances in probe tests (Fig. 2.2 and 2.4), these rats exhibited normal learning curves during training (see Fig. 2.2B and 2.4B). These findings suggest that hippocampal LTD is not required for the acquisition of spatial memory. To further test this hypothesis, we performed a probe test
immediately after training (Fig. 2.5A). We found that the preference for $Q_{\text{test}}$ over $Q_{\text{opp}}$ in the post-training probe test (performed 30 s after the last training trial) was well-preserved in both Ro- and Tat-GluR$_{3Y}$ peptide-treated rats (Fig. 2.5A). To address the potential effect of LTD inhibition on visual and sensorimotor functions (Saucier and Cain, 1995), we examined the impact of LTD inhibitors on performance in a visible platform version of MWM. We found that neither Ro nor Tat-GluR$_{23Y}$ peptide affected the ability of rats to find the visible platform either during the training (day 1, Fig. 2.5B) or retrieval phase of the task (day 2, Fig. 2.5C). Thus, hippocampal LTD is not necessary for the acquisition of spatial memory.

2.3.5 Hippocampal LTD is necessary for the consolidation of LSM

Pre-training injection of LTD inhibitors likely affects the acquisition and consolidation of spatial memory. Since we found that the acquisition of spatial memory does not require hippocampal LTD, our findings strongly suggest a role for this plastic change in consolidation of LSM. To test this directly, we examined the effect of post-training intra-hippocampal injection of Tat-GluR$_{23Y}$ peptide on LSM formation. Tat-GluR$_{23Y}$ peptide was bilaterally injected into the dorsal hippocampi within 5 mins after the last training trial (Fig. 2.6A). Direct intra-hippocampal injection of LTD inhibitors results in a faster blockade of LTD than is achieved with systemic injection, allowing for examination of the potential requirement of hippocampal LTD in rapid consolidation of spatial memory. We found that while the $Q_{\text{test}}$ preference of vehicle-treated rats remained intact ($Q_{\text{test}}$ 19.8 ± 1.0 s vs. $Q_{\text{opp}}$ 11.3 ± 0.7 s; n = 6, p = 0.003, Fig. 2.6B), no $Q_{\text{test}}$ preference was observed in Tat-GluR$_{23Y}$ peptide-treated rats ($Q_{\text{test}}$ 11.9 ± 1.4 s vs. $Q_{\text{opp}}$ 15.8 ± 1.2 s; n = 7, p = 0.120). In addition, spatial memory formation remained intact in
Figure 2.5 Acquisition of spatial memory does not require hippocampal LTD

(A) Rats received pre-training injection of saline, Ro, or Tat-GluR_{3Y} peptide and were given a single probe test 30s after the last training trial. Note that the preference for Q_{test} in the probe test was observed in rats from all treatment groups. (B) Additional groups of rats that received pre-training injection of saline, Ro, or Tat-GluR_{3Y} peptide were trained with 8 trials to find a visible platform on day 1. Histograms depict the change in mean escape latency from the first 4 to the last 4 training trials. The mean latencies to find the visible platform in a single trial conducted 24 hrs after training are summarized in the histogram in panel (C). Note that LTD inhibitors did not affect performance in this visible platform task. * $p < 0.05$ between time in Q_{test} and time in Q_{opp}, paired Student’s t-test in (A); * $p < 0.05$ between the first 4 and the last 4 trials, paired Student’s t-test in (B).
A. **Probe test immediately after training**

- **Saline**
  - Time in Quadrant (s): Q$_{opp}$, Q$_{last}$

- **Ro**
  - Time in Quadrant (s): Q$_{opp}$, Q$_{last}$

- **Tat-GluR2$_{yr}$**
  - Time in Quadrant (s): Q$_{opp}$, Q$_{last}$

B. **Visible platform training day 1**

- **Saline**
  - Escape latency (s): Trial 1-4, Trial 5-8

- **Ro**
  - Escape latency (s): Trial 1-4, Trial 5-8

- **Tat-GluR2$_{yr}$**
  - Escape latency (s): Trial 1-4, Trial 5-8

C. **Visible platform training day 2**

- **Saline**
  - Escape latency (s):

- **Ro**
  - Escape latency (s):

- **Tat-GluR2$_{yr}$**
  - Escape latency (s):
Figure 2.6 Consolidation of spatial memory was abolished by Tat-GluR23Y peptide

(A) Vehicle (saline), Tat-GluR23Y peptide and scrambled peptide were bilaterally injected into the dorsal hippocampi of MWM-trained rats within 5 mins of the end of the final training trial. Probe tests were conducted 24 hrs after the injection. (B) We found that Tat-GluR23Y peptide but not scrambled peptide abolished the preference for $Q_{test}$ in probe tests. * $p < 0.05$ between time in $Q_{test}$ and time in $Q_{opp}$, paired Student’s t-test.
scrambled peptide treated rats ($Q_{\text{test}}$ 18.6 ± 1.1 s vs. $Q_{\text{opp}}$ 12.1 ± 0.7 s; n = 7, p = 0.007).

Taken together, our findings strongly suggest that hippocampal LTD is required for the rapid consolidation of spatial memory.

2.3.6 LTD inhibitors do not affect the retrieval of LSM

Next, we examined if hippocampal LTD is required for LSM retrieval. We injected MWM trained rats with either Ro or Tat-GluR23Y peptide 30 mins before the probe test done 2 hrs after training (Fig. 2.7A). We found that the $Q_{\text{test}}$ preference was well preserved in Ro- and Tat-GluR23Y peptide-treated rats when these inhibitors were administered only before the probe test (Fig. 2.7B). Thus, hippocampal LTD is unlikely required for the retrieval of LSM.

2.3.7 Hippocampal LTP is unlikely required in different phases of LSM formation

We showed that pre-training injection of NVP did not affect LSM formation (Fig. 2.2), thus it is unlikely that this particular form of hippocampal CA1 LTP contributes to the acquisition and consolidation of spatial memory. However, hippocampal LTP may be required for other phases of LSM formation such as retrieval of spatial memory. Nonetheless, we found that pre-probe test administration of NVP failed to affect probe test performance (Fig. 2.8).
Figure 2.7 Retrieval of spatial memory does not require hippocampal LTD

(A) MWM-trained rats were injected i.p. with saline, Ro, or Tat-GluR2ΔY peptide 30 mins before probe tests. (B) Histograms show probe test performance. Note that neither Ro nor Tat-GluR2ΔY peptide affected the preference for Qtest in probe tests. * p < 0.05 between time in Qtest and time in Qopp, paired Student’s t-test.

(B) Histograms show probe test performance. Note that neither Ro nor Tat-GluR2ΔY peptide affected the preference for Qtest in probe tests. * p < 0.05 between time in Qtest and time in Qopp, paired Student’s t-test.
Figure 2.8 Retrieval of spatial memory does not require LTP

(A) NVP was i.p. injected into MWM-trained rats 30 mins before probe tests. (B) Histogram shows that the preference for $Q_{test}$ in probe test was intact in NVP-treated rats.

*p < 0.05* between time in $Q_{test}$ and time in $Q_{opp}$, paired Student’s t-test.
2.4 Discussion

In this study, we have used pharmacological approaches to dissect the contribution of bidirectional hippocampal synaptic plasticity to LSM formation and observed that: 1) NR2A antagonist NVP selectively prevented LTP formation in freely moving adult rats. In addition, we found that hippocampal LTD, which is inducible in freely moving adult rats, was abolished by either a NR2B antagonist Ro that inhibits LTD induction or Tat-GluR23Y peptide, a synthetic peptide that disrupts LTD expression through blocking AMPAR endocytosis. 2) Preventing LTP formation in freely moving rats using NVP did not affect LSM formation. 3) Both Ro and Tat-GluR23Y peptide, which prevent LTD formation through different mechanisms, impaired LSM formation. In particular, our results suggest that LTD is likely required for the consolidation of spatial memory. To our knowledge, these findings provide the first evidence in support of a requirement for hippocampal LTD in LSM formation in genetically unaltered adult rats.

The functional contribution of different NR2-containing NMDARs in bidirectional hippocampal synaptic plasticity has been the focus of extensive debates in the last few years. Based on our in vitro findings that specific NR2A and NR2B antagonists inhibited hippocampal LTP and LTD, respectively (Liu et al., 2004a), and the well known roles for the NR2 subunit to determine the gating (Gielen et al., 2009) and signaling properties (Bayer and Schulman, 2001) of NMDAR, we favor a model in which the polarity of hippocampal synaptic plasticity is largely determined by the subunit composition of NMDARs. This model has received both support (e.g. (Salter and Kalia, 2004; Wong et al., 2007; Zhu et al., 2005)) and challenges ((Beazely et al., 2009;
Morishita et al., 2007)), with the major focus of research being to test if LTP and LTD are dictated by different NR2-containing NMDARs. Given that NVP and Ro cross the blood brain barrier (Fox et al., 2006), we further tested the NR2 model by asking if these antagonists can differentially inhibit hippocampal LTP and LTD \textit{in vivo} using either anesthetized (Fox et al., 2006) or freely moving rats (present study). In line with our prediction, we found that NVP and Ro were able to selectively inhibit hippocampal LTP and LTD, respectively, in behaving rats. These findings not only provide additional support for the importance of NMDAR molecular composition in determining the polarity of synaptic plasticity, but also illustrate the potential utility of NR2A and NR2B antagonists in probing functional roles of LTP and LTD in behaving animals. Indeed, we found that preventing LTD by Ro impairs LSM formation. The requirement of hippocampal LTD in the formation of LSM is supported by our finding that LSM was disrupted by Tat-GluR23Y peptide, an antagonist that inhibits LTD expression. In future, NR2 specific antagonists could be used to probe the functional contribution of LTP and LTD in other forms of long-term memory (e.g. recognition memory) and behaviors in adult rats.

As administration of NVP before training (Fig. 2.2) or before probe test (Fig. 2.8) did not affect LSM retrieval in MWM-trained rats, our findings suggest that it is unlikely that hippocampal LTP is essential for LSM formation. Interestingly, previous attempts to specifically disrupt hippocampal LTP have revealed similar findings. For instance, although knocking down the GluR1 subunit of AMPAR abolishes hippocampal LTP formation, GluR1 KO mice have intact LSM (Zamanillo et al., 1999). Similarly, NR2A knock-out or NR2A carboxyl-tail knock-out mice, which exhibit impaired LTP (Kohr et
al., 2003; Sakimura et al., 1995), do not display major deficits in LSM formation (Bannerman et al., 2008). Notably, both GluR1 and NR2A KO mice perform poorly in spatial working memory tasks (Bannerman et al., 2008; Reisel et al., 2002). NVP provides a powerful tool to examine the functional requirement of hippocampal LTP for spatial working memory formation in genetically unaltered animals.

If NMDAR-dependent hippocampal LTP is not necessary for LSM formation, LTD, another NMDAR-mediated cellular process, could be responsible for the impact of NMDAR blockade on LSM formation in MWM (Morris et al., 1986). Consistent with this hypothesis, our findings support the involvement of hippocampal LTD in the consolidation of long-term spatial memory. First, we found that Ro, a NR2B antagonist that prevents hippocampal LTD but not LTP induction (Fig. 2.1), impaired probe test performance in MWM (Fig. 2.2). Second, LSM formation was also affected by Tat-GluR2_3Y peptide (Fig. 2.4), which inhibits an LTD expression mechanism, namely AMPAR endocytosis. Third, direct intra-hippocampal injection of Tat-GluR2_3Y peptide after spatial learning hampered LSM formation (Fig. 2.4). In addition to providing evidence in support of a role for NMDAR-dependent hippocampal LTD in LSM formation, our findings could explain the dilemma that LSM formation can be disturbed by blocking both LTP and LTD using a subunit nonspecific antagonist APV (Morris et al., 1986) but not by directly targeting LTP using either genetic (Bannerman et al., 2008; Zamanillo et al., 1999) or pharmacological approaches (data from present study).

Although we showed that administering a specific NR2B antagonist Ro during MWM training impaired LSM formation, previous attempts to study the impact of NR2B blockade on MWM performance did not obtain similar results. For instance, using a
multiple-day training protocol, pre-training administration of CP-101,606 (Guscott et al., 2003) or Ro63-1908 (Higgins et al., 2003) did not affect the retrieval of LSM in probe tests performed at 24 hrs after last training trial. Differences in the training procedures (multiple-day training versus single-day training in the present study) and different antagonists used between previous and present studies may account for the inconsistencies in findings. More importantly, the sufficiency of the doses of NR2B antagonist used in previous studies to prevent hippocampal LTD remains in question, since no in vivo recording of LTD was performed in these studies.

While the requirement of hippocampal LTD in LSM is initially surprising, LTD has already been highly implicated in learning and memory (Bear and Abraham, 1996; Kemp and Manahan-Vaughan, 2007). For example, it has been shown that hippocampal LTD in behaving animals can be facilitated by exposure to novel objects (Manahan-Vaughan and Braunewell, 1999), which suggests a functional role for LTD in detecting novel information during learning. The functional link between LTD and novelty detection is further supported by findings from LTD-null mice that lack a transcription factor, serum response factor (SRF), and fail to habituate to novel objects in an object recognition task (Etkin et al., 2006). It is very interesting to note that SRF knockout mice also display poor LSM formation in MWM, which parallels the impact of acute blockade of LTD formation in MWM consolidation observed in the present study.

How hippocampal LTD contributes to the consolidation of spatial memory is not clear. Although LTD reduces the strength of glutamate synapses, it shares several properties of LTP (Bliss and Collingridge, 1993), which include input specificity, cooperativity, and associativity (Dudek and Bear, 1992). These properties make LTD a
potential ‘Hebbian’ mechanism for information computation and storage in the hippocampus. Since hippocampal synapses are spontaneously active, consolidation of spatial information may be achieved by depressing a specific subset of these active synapses in a long-term manner through a LTD mechanism. Indeed, \textit{in vivo} findings from Manahan-Vaughan’s group showed that LTD and LTP are facilitated by exposing rats to novel objects and empty space, respectively (Kemp and Manahan-Vaughan, 2004), which suggest that LTD and LTP encode different types of information during spatial learning. Blocking hippocampal LTD by either Ro or Tat-GluR23Y peptides could therefore disturb the storage of spatial information and lead to LSM impairment.

In conclusion, our findings support the importance of hippocampal LTD, but not LTP, in LSM formation in freely-moving adult rats. The specific inhibitors we used to manipulate different forms of hippocampal plasticity provide important tools for further dissecting the contribution of LTP and LTD in other hippocampal functions.
2.5 References


Hayashi, T., and Huganir, R.L. (2004). Tyrosine phosphorylation and regulation of the
AMPA receptor by SRC family tyrosine kinases. J Neurosci 24, 6152-6160.


3. SPECIFIC MODULATION OF HOMOMERIC GLUR1 RECEPTORS BY p97 (VCP)

3.1 Introduction

Glutamate is the primary excitatory neurotransmitter in the brain, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type glutamate receptors mediate most fast synaptic transmission. Regulated postsynaptic trafficking of AMPA receptors is a major mechanism contributing to synaptic plasticity. Structurally, AMPARs are tetrameric complexes assembled by combining four homologous subunits: GluR1-GluR4 (Collingridge et al., 2004). Each subunit contains a large extracellular N-terminal domain, three trans-membrane spanning domains (TM1, TM3 and TM4), a re-entry hairpin loop (M2) that forms the pore-lining region, an extracellular loop (S2) between TM3 and TM4, and an intracellular C-terminal domain. The subunit composition of AMPARs determines channel gating kinetics and trafficking properties. Due to the RNA editing of the critical glutamine 607 residue (Q607) to arginine (R607) in M2 of GluR2, GluR2-containing AMPARs are Ca\(^{2+}\)-impermeable, electrically linear or outwardly rectificated current/voltage (I/V) relationship and resistant to block by polyamines, while GluR2-lacking AMPARs are Ca\(^{2+}\)-permeable, electrically inward rectificated, and blocked by polyamines.

The dimerization of AMPAR subunits is initiated by the co-translational interaction of N-terminals in the endoplasmic reticulum (ER) and the underlying mechanism is poorly understood (Kuusinen et al., 1999). In hippocampal pyramidal cells, the AMPA receptor subtypes that predominate are composed of GluR1/2- and GluR2/3-

---

A version of this chapter will be submitted for publication. Ge, Y., Lu, J., Liu, L., Wong, T.P., Wu, D., Cho, T., Lin, S., Kast, J, and Wang, Y.T. Specific modulation of homomeric GluR1 receptors by p97 (VCP).
containing receptors (Wenthold et al., 1996). However, recent work (Ju et al., 2004; Kumar et al., 2002; Terashima et al., 2004) suggests that principal neurons contain substantial intracellular reserve pools of GluR2-lacking AMPARs (GluR1 homomers), which may be synaptically incorporated under specific conditions. By comparing several properties of AMPARs before and after the induction of LTP in hippocampal slices, Plant and colleagues reported that LTP may involve the insertion of GluR2-lacking native AMPARs (likely homomeric GluR1) during the first 20 min of the LTP expression, and that calcium influx through these GluR2-lacking receptors is a prerequisite for the full expression of LTP (Plant et al., 2006). These results are in a good agreement with several studies that provide strong evidence for such a rapid insertion of calcium permeable homomeric GluR1 AMPARs under various physiological and pathological conditions (Cull-Candy et al., 2006; Isaac et al., 2007) (but also see recent disputing results from Nicoll’s group (Gray et al., 2007)). However, the underlying mechanisms, in which the formation and trafficking of homomeric GluR1 receptors are modulated, are still unknown.

Interestingly, the critical role for AMPA receptors trafficking in synaptic plasticity has motivated intensive studies of interacting protein partners that modulate channel properties and trafficking of receptors. One of these proteins is a type II AAA (ATPase associated with a variety of activities) ATPase, NSF (N-Ethylmaleimide sensitive factor), which interacts with C-terminal of GluR2 and is required to maintain the expression of AMPARs at synapses (Lee et al., 2002; Nishimune et al., 1998; Noel et al., 1999). Using co-immunoprecipitation (co-IP) combined with mass spectrometric analysis, we firstly identified a new AMPAR-associated protein named p97. p97 is also
called valonsin-containing protein (VCP), which is another type II AAA ATPase that shares a high sequence homology with NSF (Peters et al., 1990). p97 is an essential protein, conserved throughout evolution from archaea to mammals (Dreveny et al., 2004), and has been shown to mediate many cellular activities such as ubiquitin-proteasome-mediated protein degradation (Kaneko et al., 2003; Meyer et al., 2000), membrane fusion (Latterich et al., 1995; Meyer et al., 2002), ER-associated degradation (Ye et al., 2001, 2003), transcription activation (Asai et al., 2002; Dai et al., 1998), and cell cycle control (Dai and Li, 2001; Yamada et al., 2000) etc. Moreover, loss of p97 function in cells by the overexpression of a dominant-negative p97 or by p97 siRNA treatment leads to cell death (Hirabayashi et al., 2001; Wojcik et al., 2004). However, the function of p97 in neurons is still unclear, even though several studies show that a mutation in p97 is related to neuronal degeneration diseases (Gitcho et al., 2009; Neumann et al., 2007). In the present work, we provided evidence that p97 specifically interacts and modulates the formation and trafficking of homomeric GluR1 receptors, which may play an important role in mediating the postsynaptic insertion of homomeric GluR1 receptors in the early stage of LTP.
3.2 Materials and methods

3.2.1 Plasmids construction

The GluR1-HA, GluR2-HA, and GluR1/R2C plasmids were generated as previously prescribed (Man et al., 2000). R1Δ809-889, R1Δ594-889, R1Δ542-889 were generated by mutating the corresponding codon to the stop codon with a Quick-Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). R1Δ510-541, R1Δ121-240, and R1Δ416-503 were generated by PCR based mutagenesis to delete the corresponding domain with a Quick-Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). For switching N-terminal of GluR1 with N-terminal of GluR2, HindIII sites were generated by point mutation in the N-terminals of GluR1-HA and GluR2-HA immediately before the first transmembrane domains. After digestion by HindIII, the resulting backbone of HA-GluR1 was ligated to the GluR2 N-terminal. GST-R1N and GST-R2N were generated by PCR amplification of GluR1 NT (4-503) and GluR2 NT (1-528) and inserted into EcoRI and SalI sites of pGEX-4T-1 vector (Amersham, Piscataway, NJ, USA). GluR1 4-374, 376-503, 4-120, 121-240, 241-374, 416-503, 376-456 and 456-503 were generated by amplification of the corresponding sequence and inserted into EcoRI and SalI sites of pCMV-HA vector (Clontech, Mountain View, CA, USA). his-p97 was provided by Dr. Graham Warren (Yale University School of Medicine). Furthermore, p97 plasmide expressed in mammalian cells was generated by PCR amplification of p97 and inserted into NotI and BamHI sites of pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). The restriction enzymes were purchased from New England Biolabs Inc. (Pickering, ON, Canada). All the
constructions were confirmed by sequencing. The details of construction please refer the supplementary materials.

3.2.2 Cell line culture and transfection

COS-7 or HEK293 cells were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the recommended manufacture’s protocols. Fourty-eight hours after transfection, cells were used for the immunoprecipitation, immunofluorescence, or electrophysiological recordings described below.

3.2.3 Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were carried out essentially as previously reported (Man et al., 2000). Hippocampal tissue, cultured hippocampal neurons or transfected COS-7 cells were homogenized in ice-cold RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.5% Deoxycholic Acid Sodium, and a cocktail of protease inhibitors (Roche Applied Science, Mannheim, Germany). After incubation on ice for 30 min, the homogenates were centrifuged at 14,000 rpm at 4°C for 15 min. The supernatants were collected and the total protein concentrations were measured using protein assay reagents (Bio-Rad Laboratories, Hercules, CA, USA). For the immunoprecipitation experiments, 1 mg cell lysates were incubated with anti-GluR1 (4 μL serum, lab raised rabbit polyclonal antibody against C-terminal 816–889), anti-GluR2 (4 μL serum, lab raised rabbit polyclonal antibody against C-terminal 833–883), or anti-HA (1 μg, Roche Applied Science, Mannheim, Germany) in 1 mL of RIPA buffer for 4 h at 4°C. Sixty mililitres Protein A–sepharose (Amersham,
Piscataway, NJ, USA) was added to the mixture and incubated overnight. The complex
was isolated by centrifugation (500×g, 2 min) and washed twice with washing buffer
(500 mM NaCl, 1% Triton X-100, 50 mM Tris–HCl, pH 7.4) and twice with PBS. The
precipitated proteins were eluted from the sepharose beads by boiling in 2× sample buffer
at 95 °C for 5 min. Thirty µg lysate were used as control for total protein expression
level.

Proteins eluted from the beads or total lysates were subjected to a 10% or a
gradient 4-15% (Bio-Rad Laboratories, Hercules, CA, USA) sodium dodecyl sulfate–
polyacrylamide gel and were transferred to a polyvinylidene difluoride membrane. The
membrane was blocked using 5% milk for 1 h at room temperature, immunoblotted,
sequentially stripped, and re-probed on the same blot by using anti-p97 (Fitzgerald,
Concord, MA, USA; 1:1000), anti-GluR1 (lab raised rabbit polyclonal antibody against
C-terminal 816–889; 1:2500), anti-GluR2 (Chemicon, Temecula, CA, USA; 1:1000), and
anti-HA (Roche Applied Science, Mannheim, Germany; 1:1000) antibodies. Blots were
developed using enhanced chemiluminescence detection (Amersham, Piscataway, NJ,
USA) and imaged with the Bio-Rad gel imaging system (Bio-Rad Laboratories, Hercules,
CA, USA). Protein band intensities were quantified with Quantity One® software (Bio-
Rad Laboratories, Hercules, CA, USA).

3.2.4 Mass spectrometric analysis

Proteins participated by co-immunoprecipitation with anti-GluR1 or anti-GluR2
antibody were subjected to SDS-PAGE, and subsequently stained using a ProteoSilver™
Silver Stain Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s
protocol. Protein bands of interest were excised from the SDS-PAGE gel and subjected
to in-gel trypsinization as previously described (Shevchenko et al., 1996). Digestions were carried out overnight at 37 °C with sequencing grade trypsin (Promega, Madison, WI, USA). Peptides were extracted, dried down and then reconstituted in 0.1% TFA and desalted with μ-C18 ZipTips (Millipore, Bedford, MA, USA). Peptides bound to the ZipTip were eluted out in 50:50 (v/v) ACN/0.1% HAc and mixed with saturated solution of CHCA (in 30:70 ν/v ACN/0.1% TFA) in the ratio of matrix to analyte of 1:1 (v/v). Approximately 0.8 μL of the mixture was deposited onto a sample plate and air dried. MALDI–TOF MS was performed on 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) using positive ion reflector mode. The peptide spectra were internally calibrated with bradykinin and ATCH peptides (Sigma, Oakville, ON, Canada) and processed with the 4700 Explorer software. MS data was analyzed and searched for protein identification using the MASCOT search engine (http://www.matrixscience.com).

3.2.5 GST pull down assay

GST-R1N, GST-R2N and his-p97 were expressed in BL21 Escherichia coli and purified from bacterial lysates according to the manufacturer’s protocols (Pharmacia Biotech, Piscataway, NJ, USA). Equal amounts of glutathione sepharose conjugated GluR1NT and GluR2NT (adjusted by commosie blue staining after resolved by SDS-PAGE) were mixed with 0.5 μg purified his-p97 at 4 °C overnight. After washing 4 times with PBS, the participated protein complexes were boiled in 2× sample buffer at 95 °C for 5 min. Proteins were resolved by SDS-PAGE, and probed with the corresponding antibodies as described in Immunoprecipitation and Western Blotting.
3.2.6 Biotinylation

Biotinylation of cell surface proteins in COS-7 cells were done following the protocol prescribed previously (Yu et al., 2008). Briefly, cells were washed 3 times with ice-cold ECS (140 mM NaCl, 1.3 mM CaCl₂, 5.4 mM KCl, 1 mM MgCl₂, 25 mM HEPES, 33 mM glucose; pH 7.4), and incubated in the ECS containing 1 mg/ml EZ-link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) at 4 °C for 30 min. Afterwards, the remaining active biotin was quenched by washing 3 times with ice-cold ECS containing 100 mM glycine. After homogenization in lysate buffer, the biotinylated proteins were isolated using streptavidin-conjugated sepharose beads (Sigma-Aldrich, St. Louis, MO, USA), eluted from the beads, resolved by SDS-PAGE, and immunoblotted with the corresponding antibodies as described in Immunoprecipitation and Western Blotting.

3.2.7 Immunofluorescent microscopy

COS-7 cells were plated onto poly-D-lysine-coated glass coverslips set in 24 well culture dishes and transfected with 1 μg of the plasmid of interest. Forty-eight hours after transfection, surface GluR1 were live labeled with anti-HA antibody (Roche Applied Science, Mannheim, Germany; 1:1000) for 30 min at 4 °C followed by fixation with 4% paraformaldehyde in PBS for 15 min at room temperature. Surface GluR1 was visualized with an Alexa Fluor 555 anti-rat IgG antibody (Invitrogen, Carlsbad, CA, USA; 1:500). Afterwards, then cells were permeabalized with PBS containing 0.1% Triton X-100 for 3 min. The internal GluR1 was labeled with anti-HA antibody (Roche Applied Science, Mannheim, Germany; 1:1000), and visualized with an Alexa Fluor 488 goat anti-rat IgG antibody (Invitrogen, Carlsbad, CA, USA; 1:500). Images were collected using a LEICA DMIRE2 microscope. For quantification of surface GluR1, the
pseudocolor intensity of surface and internal GluR1 from randomly selected 7-10 cells of each coverslip were analyzed by Image J software to a average ratio (surface vs. internal). The data for 6-8 coverslips of each group were compared using Student’s t-test.

### 3.2.8 Electrophysiological recording in HEK 293 cells

Whole-cell recordings were performed 2 days after transfection at room temperature (20–22°C). Whole-cell currents were recorded at a holding potential of -60 mV unless indicated elsewhere. The series resistance in these recordings varied between 6 to 8 MΩ. The patch electrode solution contained the following (mM): Cs methane sulphonate, 130; EGTA, 0.5; Mg.ATP, 4; HEPES, 10; Na.GTP, 0.3; QX314.Br, 5; NaCl, 8; spermine 0.1 (pH 7.25); and osmolarity between 280 to 290 mosmol⁻¹. The extracellular (perfusion or bathing) solution was of the following composition (mM): NaCl, 130; CaCl₂, 2; KCl, 5; MgCl₂, 2; HEPES, 10; glucose, 10; and Sucrose, 10 (pH 7.4); and osmolarity between 300 and 310 mosmol⁻¹. Rapid application/removal of glutamate (1 mM) or kainic acid (50 μM) was performed using a computer-controlled multi-barrel fast perfusion system (Warner Instruments, Hamden, CT, USA).

Whole cell currents were recorded using a MultiClamp 700A amplifier (Molecular Devices, Sunnyvale, CA, USA). Records were filtered at 2 kHz and acquired with pCLAMP 10 program (Molecular Devices, Sunnyvale, CA, USA). Peak amplitude, 20-80% rise time, desensitization and relative current (ratio of non-desensitizing current vs. peak current) were analyzed using Clampfit 10.2 software (Molecular Devices, Sunnyvale, CA, USA). Desensitization of AMPARs was characterized by time constants derived from mono- exponential fits to the decay phase.
3.2.9 Hippocampal neuron culture, LTP and LTD induction, and transfection

Medium-density hippocampal neurons were prepared from E18/19 rat embryos and maintained in Neurobasal medium containing B-27 supplement (Invitrogen, Carlsbad, CA, USA) as described before (Passafaro et al., 2001). Fourteen days after plating, chemical LTP or LTD was induced using the protocol previously described (Beattie et al., 2000; Lu et al., 2001b). After washing 3 times for 10 min each time with ECS, neurons were stimulated by application of glycine (200 μM; sucrose 100 mM; Strychnine 5 μM; 3 min) or NMDA (10 μM; 3 min) in Mg-free ECS to induce LTP or LTD, respectively. The cell lysates were collected 10 min after stimulation unless indicated elsewhere. The p97-GluR1 complex was co-immunoprecipitated using anti-GluR1 antibody, and immunoblotted with the corresponding antibodies as described in Immunoprecipitation and Western Blotting.

Neurons were transfected at 12-14 days in vitro (DIV) using ProFection® Mammalian Transfection System (Promega, Madison, WI, USA) following the protocol described before (Jiang et al., 2004), and used for electrophysiological recording at 2-3 days post-transfection (DIV14-17).

3.2.10 Recordings of miniature excitatory postsynaptic currents in cultured hippocampal neurons

Whole-cell recordings were made from the cultures 14–17 days after plating. Recordings were performed at room temperature (20–22°C), each lasting from 20 to 50 min. The series resistance in the recordings varied between 6 to 8 MΩ, and recordings
where series resistance varied by more than 10% were rejected. No electronic compensation for series resistance was employed.

The patch electrode solution contained the following (mM): Cs methane sulphonate, 130; EGTA, 0.5; Mg.ATP, 4; HEPES, 10; Na.GTP, 0.3; QX314.Br, 5; and NaCl, 8 (pH 7.25); and osmolarity between 280 to 290 mosmol$^{-1}$. The extracellular (perfusion or bathing) solution (ECS) was of the following composition (mM): NaCl, 140; CaCl$_2$, 1.3; KCl, 5.4; MgCl, 1; HEPES, 25; glucose, 33; TTX, 0.0005; and bicuculline methiodide, 0.02 (pH 7.4); and osmolarity between 310 and 320 mosmol$^{-1}$. Each cell was continuously superfused (1 ml/min) with this solution from a single barrel of a computer-controlled multibarreled perfusion system. Solutions supplemented with glycine were applied from an alternative barrel.

mEPSCs were recorded using an MultiClamp 700A amplifier (Molecular Devices, Sunnyvale, CA, USA). Records were filtered at 2 kHz, and acquired with pCLAMP 10 program (Molecular Devices, Sunnyvale, CA, USA). Cells that demonstrated a change in “leak” current of more than 10% (usually less than 10 pA) were rejected from the analysis. The trigger level for detection of events was set approximately 3 times higher than the baseline noise. Inspection of the raw data was used to eliminate any false events, and mEPSCs recorded in 3 min period of each condition were analyzed using Mini 6.0 software (Synaptosoft, Decatur, GA, USA).
3.2.11 Statistical analysis

Values are expressed as Mean ± SEM, and analyzed using a Student’s t-test for comparison between two groups and ANOVA followed by post hoc (Fisher LSD) tests for comparisons among multiple groups. Statistical significance is defined as p < 0.05.
3.3 Results

3.3.1 Mass spectrometric analysis of novel AMPAR associated proteins

In order to investigate novel AMPAR associated proteins, we raised antibodies against the C-terminal of GluR1 or GluR2 subunits, the most expressed AMPAR subunits in hippocampus. To determine the specificity of these antibodies, they were used to immunoprecipitate GluR1-HA or GluR2-HA, which were heterogeneously expressed in COS-7 cells, followed by Western Blotting with an anti-HA antibody (Fig. 3.1A). These two antibodies showed very good selectivity without any cross-reaction between GluR1 and GluR2 subunits (Fig. 3.1A). We used these antibodies to immunoprecipitate the AMPAR complex from the homogenates of the rat hippocampus. Besides GluR1 and GluR2, a clear protein band was found only in the GluR1 antibody precipitates (Fig. 3.1B). Mass spectrometric analysis identified p97 as a potential protein with the highest probability based Mowse Score (240) and a peptide sequence coverage of 54% (Table 3.1).

p97 is a member of the type II AAA ATPase family, which are characterized by the presence of two conserved ATPase domains (Confalonieri and Duguet, 1995; Neuwald et al., 1999). p97 acts as a molecular chaperone that binds to adaptor proteins and transfers energy from ATP hydrolysis through the adaptor to mediate many cellular activities. To further test the findings from mass spectrometry, we repeated the co-immunoprecipitation experiment, and probed it with an anti-p97 antibody. The results confirmed that p97 could be co-immunoprecipitated by anti-GluR1 only and not anti-GluR2 antibody (Fig. 3.2A).
Figure 3.1 Co-immunoprecipitation to identify novel AMPAR associated proteins

(A) Specificity test of lab-raised anti-GluR1 and anti-GluR2 antibodies. HA-tagged GluR1 and GluR2 expressed in COS-7 cells were immunoprecipitated with lab-raised anti-GluR1 and GluR2 antibodies, and immunoblotted by an anti-HA antibody, which detected both GluR1 and GluR2.

(B) Co-immunoprecipitation to identify novel AMPAR-associated proteins. Homogenates of the rat hippocampus were co-immunoprecipitated with anti-GluR1 or GluR2 antibodies to look for novel AMPAR-associated proteins. The precipitated proteins were detected using silver staining after resolved on a 4-15% gradient SDS-PAGE gel. The protein band highlighted by the arrow head was excised for mass spectrometric analysis.
<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Peptide mass</th>
<th>m/z</th>
<th>Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIVDEAINEDNSVVSLSQPK</td>
<td>2169.12</td>
<td>2170.12</td>
<td>26-45</td>
</tr>
<tr>
<td>MDELQLFR</td>
<td>1050.52</td>
<td>1051.52</td>
<td>46-53</td>
</tr>
<tr>
<td>GDTVLIK</td>
<td>744.44</td>
<td>745.43</td>
<td>54-60</td>
</tr>
<tr>
<td>NNLVR</td>
<td>770.45</td>
<td>771.43</td>
<td>90-95</td>
</tr>
<tr>
<td>LGDVISIQPCPDVK</td>
<td>1539.80</td>
<td>1540.81</td>
<td>96-109</td>
</tr>
<tr>
<td>KGDIFLVR</td>
<td>946.56</td>
<td>947.57</td>
<td>148-155</td>
</tr>
<tr>
<td>GDIFLVR</td>
<td>818.46</td>
<td>819.48</td>
<td>149-155</td>
</tr>
<tr>
<td>EDEEESLNEVGYDDIGGCR</td>
<td>2184.88</td>
<td>2185.94</td>
<td>192-210</td>
</tr>
<tr>
<td>EMVELPLR</td>
<td>985.53</td>
<td>986.54</td>
<td>218-225</td>
</tr>
<tr>
<td>GILLYGPPGTGK</td>
<td>1171.66</td>
<td>1172.66</td>
<td>240-251</td>
</tr>
<tr>
<td>AVANETGAFSSLINGPEIMSK</td>
<td>2255.13</td>
<td>2256.15</td>
<td>257-277</td>
</tr>
<tr>
<td>LAGESESNLR</td>
<td>1074.53</td>
<td>1075.54</td>
<td>278-287</td>
</tr>
<tr>
<td>KAFEEAEK</td>
<td>950.47</td>
<td>951.47</td>
<td>288-295</td>
</tr>
<tr>
<td>NAPAIIIFIDELDAIAPK</td>
<td>1809.99</td>
<td>1810.98</td>
<td>296-312</td>
</tr>
<tr>
<td>IVSQLTLMDGLK</td>
<td>1429.82</td>
<td>1430.83</td>
<td>324-336</td>
</tr>
<tr>
<td>FGRFDR</td>
<td>796.40</td>
<td>797.39</td>
<td>360-365</td>
</tr>
<tr>
<td>EVDIGIPDATGR</td>
<td>1241.63</td>
<td>1242.63</td>
<td>366-377</td>
</tr>
<tr>
<td>LEILQIHTK</td>
<td>1093.65</td>
<td>1094.65</td>
<td>378-386</td>
</tr>
<tr>
<td>WALSQNSPALR</td>
<td>1328.68</td>
<td>1329.69</td>
<td>454-465</td>
</tr>
<tr>
<td>ETVVEVPQVTWEDIGGLEDVKR</td>
<td>2497.27</td>
<td>2498.28</td>
<td>466-487</td>
</tr>
<tr>
<td>ELQELVQVPVEHPDK</td>
<td>1822.91</td>
<td>1823.92</td>
<td>488-502</td>
</tr>
<tr>
<td>ELQELVQVPVEHPDKFLK</td>
<td>2211.16</td>
<td>2212.16</td>
<td>488-505</td>
</tr>
<tr>
<td>FGTPSK</td>
<td>766.37</td>
<td>767.38</td>
<td>506-512</td>
</tr>
<tr>
<td>GVLFYGPPCGK</td>
<td>1250.61</td>
<td>1251.61</td>
<td>513-524</td>
</tr>
<tr>
<td>AIANECQANFISIK</td>
<td>1577.79</td>
<td>1578.80</td>
<td>530-543</td>
</tr>
<tr>
<td>GPELLTMWGESEANVR</td>
<td>1950.91</td>
<td>1951.92</td>
<td>544-560</td>
</tr>
<tr>
<td>QAAPCVLFFDELDSIAK</td>
<td>1922.94</td>
<td>1923.93</td>
<td>568-584</td>
</tr>
<tr>
<td>GGNIGDGGGAADR</td>
<td>1115.50</td>
<td>1116.50</td>
<td>587-599</td>
</tr>
<tr>
<td>VINVQITEMDGMSTK</td>
<td>1678.83</td>
<td>1679.84</td>
<td>600-614</td>
</tr>
<tr>
<td>VINVQITEMDGMSTK</td>
<td>1806.92</td>
<td>1807.91</td>
<td>600-615</td>
</tr>
<tr>
<td>LDQLIYIPLDEK</td>
<td>1555.85</td>
<td>1556.85</td>
<td>639-651</td>
</tr>
<tr>
<td>DVIDLEFLAK</td>
<td>1048.54</td>
<td>1049.54</td>
<td>669-677</td>
</tr>
<tr>
<td>MTNGFSADLTEICQR</td>
<td>1798.80</td>
<td>1799.80</td>
<td>678-693</td>
</tr>
<tr>
<td>ESIESEIR</td>
<td>961.47</td>
<td>962.47</td>
<td>701-708</td>
</tr>
<tr>
<td>ESIESEIRR</td>
<td>1117.57</td>
<td>1118.57</td>
<td>701-709</td>
</tr>
<tr>
<td>QTNPSSMEVEEDDPVPEIR</td>
<td>2154.97</td>
<td>2155.99</td>
<td>714-732</td>
</tr>
<tr>
<td>RDHFEEAMR</td>
<td>1189.53</td>
<td>1190.55</td>
<td>733-741</td>
</tr>
<tr>
<td>RSVSDNDIR</td>
<td>1060.53</td>
<td>1061.53</td>
<td>745-753</td>
</tr>
<tr>
<td>Peptide sequence</td>
<td>Peptide mass</td>
<td>m/z</td>
<td>Residues</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------</td>
<td>--------</td>
<td>----------</td>
</tr>
<tr>
<td>SVSDNDIR</td>
<td>904.42</td>
<td>905.44</td>
<td>746-753</td>
</tr>
<tr>
<td>SVSDNDIRK</td>
<td>1032.52</td>
<td>1033.53</td>
<td>746-754</td>
</tr>
<tr>
<td>KYEMFAQTLQQSR</td>
<td>1628.80</td>
<td>1629.81</td>
<td>754-766</td>
</tr>
</tbody>
</table>
Figure 3.2 p97 selectively interacts with homomeric GluR1 AMPARs

(A) The homogenates of hippocampal slices were co-immunoprecipitated with anti-GluR1 or GluR2 antibody, and sequentially probed with anti-p97, anti-GluR1, and anti-GluR2 antibodies. p97 was co-immunoprecipitated by anti-GluR1, but not the anti-GluR2 antibody.

(B) p97 specifically interacts with GluR1 subunit. p97 was co-immunoprecipitated when cotransfected with GluR1-HA but not GluR2-HA in COS-7 cells. Please note that anti-GluR1 or anti-GluR2 cannot pull down p97 when p97 was transfected alone.

(C) p97 interacts with homomeric GluR1 receptors. With an increase in the expression level of GluR2, the p97-GluR1 interaction was decreased in COS-7 cells expressing equal amounts of GluR1-HA (6 μg) and p97 (6 μg), and different amounts of GluR2 (0, 1, 3, 6, and 12 μg).
3.3.2 p97 interacts with homomeric GluR1 AMPARs

The majority of AMPARs expressed in hippocampus are GluR1/GluR2 or GluR2/GluR3 heteromeric receptors (Wenthold et al., 1996). However, a small but significant portion of AMPARs are GluR1 homomeric receptors (Wenthold et al., 1996). To test whether p97 interacts with GluR1 or GluR2 or both, p97 was co-transfected with GluR1-HA or GluR2-HA in COS-7 cells. Fouty-eight hours later, the cell lysates were collected for co-immunoprecipitation using an anti-GluR1 or anti-GluR2 antibody. p97 was co-immunoprecipitated with GluR1 but not GluR2 (Fig. 3.2B), suggesting a high selectivity of p97 in the interaction with different AMPAR subunits. p97 is highly abundant in all types of cells, accounting for more than 1% of the total cellular protein (Wang et al., 2004). No p97 signal was detected when co-IPs were performed from p97 transfected cell lysate (Fig. 3.2B), which suggests that our lab-raised anti-GluR1 or GluR2 antibody does not have any artificial cross reaction with p97.

If p97 interacts with GluR1 no matter whether GluR1 is from GluR1/GluR2 heteromeric or GluR1 homomeric AMPARs, both anti-GluR1 and GluR2 antibodies should pull down p97 from hippocampus homogenates. The result is that p97 can only be co-immunoprecipitated by anti-GluR1, but not GluR2 antibody and this may be due to its selective interaction with homomeric GluR1 AMPARs. To test this idea, equal amounts of GluR1-HA (6 μg) and p97 (6 μg) were co-transfected with different amounts of GluR2 (0, 1, 3, 6, 12 μg) in COS-7 cells. With an increase in GluR2 expression, there was a dramatic decrease of p97-GluR1 interaction (Fig. 3.2C). The reduced level of interaction cannot be explained by the decrease of expression levels of p97 or GluR1, since either p97 or GluR1 was expressed constantly in each transfection condition (Fig. 3.2C).
3.2C). However, with an increase of GluR2, more GluR1 formed heteromeric receptors with GluR2 (Fig. 3.2C), thus less homomeric GluR1 AMPARs would be available to interact with p97. Taken together, p97 selectively interacts with GluR1, but not the GluR2 subunit, and only interacts with homomeric GluR1 receptors.

3.3.3 p97 interacts with multiple domains within N-terminal of GluR1

The AMPAR subunit contains a large extracellular N-terminal domain, three trans-membrane spanning domains (TM1, TM3 and TM4), a re-entry hairpin loop (M2) that forms the pore-lining region, an extracellular loop (S2) between TM3 and TM4, and an intracellular C-terminal domain (Fig. 3.3A). To investigate which part of GluR1 is involved in the interaction with p97, we designed the following constructs of GluR1 (Fig. 3.3A): R1Δ809-889 (GluR1 without C tail), R1Δ594-889 (GluR1 without TM3, S2, TM4 and C tail), R1Δ542-889 (GluR1 without M2, TM3, S2, TM4 and C tail), R1Δ510-541 (GluR1 without TM1), R2N/R1 (GluR1 with N-terminal switched by GluR2 N-terminal), and R1/R2C (GluR1 with C-terminal switched by GluR2 C-terminal). We co-transfected these plasmids with p97 in COS-7 cells, and did co-IP using anti-HA antibody 48 hours later. The interaction of p97 with GluR1 was not decreased by deletion of TM1, M2, TM3, S2, TM4 or C tail from GluR1 (Fig. 3.3B). However, switching GluR1 N terminal with GluR2 N terminal (R2N/R1) totally abolished the interaction with p97 (Fig. 3.3B), suggesting the interaction domain is within the N-terminal of GluR1. To further confirm this and to test whether the interaction is by direct binding or whether some other adaptor proteins are needed, pull-down assay was performed using GluR1 N-terminal as GST-fusion protein (GST-GluR1NT), and GST-GluR2NT to precipitate the purified and his-tagged p97 (Fig. 3.3C). Results showed that p97 specifically interacts with
Figure 3.3 The interaction domain is within the N-terminal of GluR1

(A) Scheme of the construction of GluR1 C-terminal truncation, internal deletion or switch of C- or N-terminal: R1Δ809-889 (GluR1 without C-terminal), R1Δ594-889 (GluR1 without TM3, S2, TM4 and C-terminal), R1Δ542-889 (GluR1 without M2, TM3, S2, TM4 and C-terminal), R1Δ510-541 (GluR1 without TM1), R2N/R1 (GluR1 with N terminal switched by GluR2 N-terminal), and R1/R2C (GluR1 with C terminal switched by GluR2 C-terminal).

(B) p97 interacts with N-terminal of GuR1 subunit. p97-GluR1 complex was co-immunoprecipitated with an anti-HA antibody in COS-7 cells expressing different deleted or truncated constructs of GluR1 and p97, followed by sequentially probing with anti-p97 and anti-HA (for all versions of GluR1) antibodies. The interaction of p97 with GluR1 was abolished when the GluR1 N-terminal was switched by the GluR2 N-terminal.

(C) p97 directly binds to N-terminal of GluR1 subunit. Purified his-p97 was pulled down by GST-GluR1NT or GST-GluR2NT, which were conjugated with glutathione sepharose beads, followed by sequentially probing using anti-p97 and anti-HA (for GluR1NT and GluR2NT) antibodies. Assays showed that p97 directly interacts with N-terminal of GuR1.
GluR1NT, but not GluR2NT demonstrating that the p97-GluR1 interaction is most likely a direct protein-protein binding and the interaction domain is indeed within the N-terminal of GluR1.

The N-terminal of AMPAR subunit is composed of two parts: LIVBP (leucine/isoleucine/valine-binding protein-like domain) 1-374 a.a., which is responsible for subunit dimerization; and the adjacent LBD (ligand binding domain) 375-503 a.a., which is responsible for ligand binding (Madden, 2002). In order to determine which part is involved in the interaction with p97, we firstly co-expressed 4-374 or 376-503, which has been inserted into pCMV-HA, with p97 in the COS-7 cells. A Co-IP experiment found that both 4-374 and 376-503 interact with p97 (Fig. 3.4A left). Secondly, we split 4-374 into three segments: 4-120, 121-240 and 241-374. A Co-IP experiment revealed that the most important segment for the p97-GluR1 interaction is 121-240 (Fig. 3.4A middle). Thirdly, we studied which segment within 376-503 is important for the interaction with p97. The expression level of very short segments such as 376-456 and 456-503 was quite low, which could not be detected using our detection method. However, 416-503 had a strong binding with p97, which is comparable with 376-503 (Fig. 3.4A right). Thus far we indentified at least two domains 121-240 and 416-503 that are sufficient for the interaction with p97. However, we also found a weak interaction in 241-374 with p97 (Fig. 3.4A middle). In order to differentiate the importance of 121-240 and 416-503, we designed a PCR-based deletion of 121-240 or 416-503 from wide type GluR1-HA. A Co-IP experiment after co-transfection with p97 found that deleting either segment is not enough to abolish the association (Fig. 3.4B).
Figure 3.4 Multiple domains within the N-terminal of GluR1 are involved in p97-GluR1 interaction

(A) p97 interacts with multiple domains within N-terminal of GluR1 subunit. Different segments of GluR1 NT: 4-374, 376-503, 4-120, 121-240, 241-374, 416-503, 376-456, 456-503-pCMV-HA were coexpressed with p97 in COS-7 cells. The interaction was tested by co-immunoprecipitation with an anti-HA antibody, followed by sequential probing with anti-p97 and anti-HA (for all segments of GluR1 NT) antibodies. And the cells expressing p97 alone were used as control. Please note that p97 interacts with 121-240 and 416-503 of GluR1 N-terminal. The expression levels of 376-456 and 456-503 were too low for detection.

(B) Deletion of 121-240 or 416-503 from GluR1 does not affect p97-GluR1 interaction. GluR1 wide type, GluR1Δ121-240 (deletion of 121-240), and GluR1Δ416-503 (deletion of 416-503) were cotransfected with p97 in COS-7 cells. The interaction of p97 and GluR1 deletions were tested by co-immunoprecipitation with the anti-HA antibody, followed by sequential probing with anti-p97 and anti-HA (for all forms of GluR1) antibodies.
Taken together, p97 directly binds with N-terminal of GluR1, and this interaction is dependent on multiple domains of GluR1NT. Single domain deletion is not sufficient to block this interaction. It may therefore not be possible to use a short interfering peptide to block this interaction.

3.3.4 Changes in surface expression level and channel gating properties of homomeric GluR1 AMPARs by p97

p97 shares a high sequence similarity with N-Ethylmaleimide sensitive factor (NSF), both of which belong to type II AAA ATPase family and mediate cellular morphology changes involving membrane fusion (Peters et al., 1990). NSF interacts with C-terminal of GluR2, and plays an important role in modulating GluR2-containing AMPAR trafficking (Lee et al., 2002; Nishimune et al., 1998; Noel et al., 1999). The specific interaction of p97 with homomeric GluR1 AMPARs raised the question of whether p97 modulates trafficking of this subtype of receptors. We co-transfected GluR1-HA with p97 or the empty vector as a control into COS-7 cells. Although endogenous p97 is abundantly expressed, GluR1 only interacts with co-expressed p97 (Fig. 3.5A). So whether GluR1 was co-expressed with or without p97 represents the states whether GluR1 is associated with or without p97, respectively. In order to test whether association with p97 affects trafficking of GluR1, we labeled surface proteins using biotinylatin. Bioinylated surface GluR1 was pulled down by Avidin-sepharose, and total GluR1 was immunoprecipitated by anti-HA antibody from the 1/5 cell lysates used for Avidin pull down. With the association of p97, surface expression level of GluR1 was dramatically decreased compared to GluR1 without association of p97.
Figure 3.5  Interaction with p97 decreases cell surface expression of GluR1

(A) GluR1 only interacts with co-expressed p97 but not endogenous p97 in COS-7 cells. p97-GluR1 complex was co-immunoprecipitated by anti-HA antibody when GluR1-HA was co-expressed with p97 or vector in COS-7 cells. Please note the high expression of endogenous p97 in COS-7 cells, which cannot be co-immunoprecipitated with exogenous GluR1.

(B) Biotinylation identified a decrease in cell surface expression of GluR1 by p97. Surface GluR1 was labeled by biotinylatoin in COS-7 cells expressing GluR1 + vector or GluR1 + p97. GluR1 in the 1/5 of the amount of lysates used in avidin pull down were immunoprecipitated by anti-HA antibody to control the total expression. p97 decreased cell surface expression of GluR1, as shown by Western Blot images (top) and quantification (bottom) of surface GluR1 (surface intensity of GluR1 was normalized to 1/5 total, and then normalized to control (vector)) from 6 individual experiments. * p < 0.05, Student’s t-test compared with control (vector).

(C) The decrease of cell surface expression of GluR1 by p97 was further confirmed by immunofluorescent imaging. In COS-7 cells expressing GluR1 + vector or GluR1 + p97, surface GluR1 was labeled using anti-HA antibody under non-permeant condition, and internal GluR1 was subsequently labeled with anti-HA antibody after cell permeabilization. Interaction with p97 decreased the surface expression of GluR1, as shown in the representative images (left) and quantification (right) of surface vs. internal GluR1 from at least 50 radomly selected cells from 6 coverslips. ** p < 0.01, Student’s t-test compared with control (vector).
(D) p97 decreases the peak amplitude of the current mediated by homomeric GluR1 receptors, as shown by the representative traces induced by 1 mM glutamate in the HEK 293 cells expressing GluR1 + vector or GluR1 + p97 (left), and the quantification of peak amplitude from 14-16 cell recordings (right). ** p < 0.01, Student’s t-test compared with control (vector).
(0.5907 ± 0.1147 normalized to Vec, n = 6, p = 0.03578; Fig. 3.5B). In addition, the decreased surface expression level of GluR1 is not due to the change in total expression level, since we found there is no significant change of total expression level of GluR1 (Fig. 3.5B). And this finding was further tested by immunofluorescent staining of surface and internal GluR1. Surface GluR1 was live labeled using anti-HA antibody followed by fixation and incubation with an Alexa Fluor 555 secondary antibody. Afterwards, the cell was permeablized and internal GluR1 was labeled using anti-HA antibody followed by an Alexa Fluor 555 secondary antibody. Statistical analysis of the intensity ratio of surface vs. internal GluR1 found that the association with p97 reduced the surface expression level of GluR1 (p97 0.1455 ± 0.01628 vs. Vec 0.4375 ± 0.05881, n = 6, p = 0.002087, Fig. 3.5C).

Next, we studied whether p97 can affect the channel gating properties of homomeric GluR1 receptors. Homomeric GluR1 receptor mediated currents were induced by 1 mM glutamate in HEK 293 cells expressing GluR1 and p97 or vector as a control. The association with p97 did not change the dynamics of the activation (20-80 % rise time, p97 7.357 ± 1.095 ms, n = 14, vs. Vec 7.308 ± 1.194 ms, n = 16, p = 0.9776, Fig. 3.6B) or desensitization ($\tau_{\text{desensitization}}$, p97 16.06 ± 0.9462 ms, n = 14, vs. vector 14.45 ± 1.223 ms, n = 16, p = 0.3035, Fig. 3.6C) of homomeric GluR1 receptors. However, the relative non-desensitizing current was significantly increased with the association of p97 (ratio of non-desensitizing current to peak current, p97 0.2157 ± 0.02705, n = 14, vs. vector 0.1432 ± 0.01680, n = 16, p = 0.03294, Fig. 3.6D). Consistent with the finding that p97 reduced the surface expression level of GluR1, the amplitude of
Figure 3.6 p97 changes the channel gating properties of homomeric GluR1 AMPARs

(A) Sample traces of the current responses of homomeric GluR1 receptors induced by 1 mM glutamate in the HEK 293 cells expressing GluR1 + p97 or GluR1 + vector.

(B) Summary of the values for 20-80% rise time, τ_{desensitization}, relative current (relative amplitude of non-desensitizing current vs. peak current) obtained from 14-16 cell recording as in (A). * p < 0.05, Student’s t-test compared with control (vector).
Peak current was also decreased with the association of p97 (p97 39.99 ± 8.401 pA, n = 14, vs. vector 106.53 ± 21.66 pA, n = 16, p = 0.009805, Fig. 3.5D).

3.3.5 p97 facilitates the formation of homomeric GluR1 AMPARs and keeps them in intracellular sites

AMPAR subunits are first assembled in the ER, which depends on luminal interaction between the N-terminal domains (Kuusinen et al., 1999). Only a small portion of AMPARs are GluR1 homomeric receptors and the majority of GluR1 forms heteromeric receptors with GluR2 in hippocampus (Wenthold et al., 1996). The fact that p97 only associates with GluR1, which forms homomeric receptors, raises the question of whether p97 affects the fate of GluR1 to form homomeric or heteromeric receptors. In order to test this, we co-transfected HA-tagged GluR1 and none-tagged GluR2 with p97 or the vector as a control. Co-IP experiments found that association with p97 decreased the formation of GluR1/GluR2 heteromeric receptors (GluR2 co-immunoprecipitated by anti-GluR1 antibody: p97 0.7738 ± 0.04339 normalized to vector, n = 8, p = 0.001236 vs. vector; GluR1 co-immunoprecipitated by GluR2 antibody: p97 0.8566 ± 0.05662 normalized to vector, n = 8, p = 0.03912 vs. vector, Fig. 3.7). This result suggests that the p97-GluR1NT interaction favors the formation of homomeric GluR1 AMPARs by inhibiting the hetero-merization between the N-terminals of GluR1 and GluR2, thereby decreasing the GluR1/GluR2 heteromeric AMPARs in the cells.

It is interesting to test whether these homomeric GluR1 AMPARs which are facilitated by association with p97 will be inserted into the cell surface. The presence of GluR2 in the AMPAR assembly determines Ca^{2+} permeability and electrical rectification.
Figure 3.7 p97 decreases the formation of GluR1/GluR2 heteromeric receptors in COS-7 cells

GluR1-HA and GluR2 were cotransfected with p97 or vector in the COS-7 cells. GluR1-GluR2 complex was co-immunoprecipitated by anti-GluR1 or anti-GluR2 antibody, separately, followed by sequential probing with anti-HA (for GluR1), anti-GluR2 and anti-p97 antibodies.

(A) Images of Western Blotting showed less of GluR2 were co-immunoprecipitated with GluR1, and less of GluR1 were co-immunoprecipitated with GluR2 when GluR1/GluR2 were co-expressed with p97 compared with control (vector).

(B) Quantification of the normalized intensity of GluR2 co-immunoprecipitated by anti-GluR1 antibody (the intensity of GluR2 was normalized to GluR1, and then normalized to the vector), or GluR1 co-immunoprecipitated by anti-GluR2 antibody (the intensity of GluR1 was normalized to GluR2, and then normalized to vector) showed a significant decrease in the formation of GluR1/GluR2 heteromeric receptors when GluR1 and GluR2 were co-expressed with p97 compared with vector. The data presented were collected from 8 individual experiments. * p < 0.05, ** p < 0.01, Student’s t-test compared with control (vector).
While GluR2-containing AMPARs are Ca\(^{2+}\)-impermeable and electrically linear or outwardly rectified, GluR2-lacking AMPARs are permeable to Ca\(^{2+}\) and exhibit an inward rectification. In order to test subunit composition of AMPARs on the cell surface, we studied I/V relationship by inducing currents with 50 μM kainic acid while holding the cells at different membrane potentials. We observed an outward rectification and inward rectification of I/V relationship when HEK 293 cells were transfected by GluR2 alone (rectification index I\(_{+60\text{mV}}\)/I\(_{-60\text{mV}}\) = 2.708 ± 0.4761, n = 8; Fig. 3.8 A, B, and C) or GluR1 alone (rectification index I\(_{+60\text{mV}}\)/I\(_{-60\text{mV}}\) = 0.2670 ± 0.1482, n = 6; Fig. 3.8 A, B, and C), respectively. However, when cells were transfected with GluR1 plus GluR2, there was an almost linear I/V relationship (rectification index I\(_{+60\text{mV}}\)/I\(_{-60\text{mV}}\) = 1.333 ± 0.3597, n = 6; Fig. 3.8 A, B, and C), suggesting both GluR2-containing and GluR2-lacking (GluR1 homomeric) AMPARs were expressed on the cell surface. Interestingly, the I/V curve was shifted to GluR2 alone when the cells expressed GluR1/GluR2 with p97 (rectification index I\(_{+60\text{mV}}\)/I\(_{-60\text{mV}}\) = 2.697 ± 0.4043, n = 5; F = 8.883, p < 0.001; post-hoc GluR1 vs. GluR2 p < 0.001; R1/R2+p97 vs. GluR2 p = 0.985; R1/R2+p97 vs. R1/R2+vector p = 0.033; R1/R2+vector vs. GluR1 p < 0.001; R1/R2+vector vs. GluR2 p = 0.018; R1/R2+vector vs. GluR1 p = 0.076; Fig. 3.8 A, B, and C), which could be due to a decrease of GluR2-lacking, or an increase of GluR2-containing AMPARs on the cell surface. To test whether p97 affected cell surface expression level of GluR1 and GluR2, we labeled surface proteins by biotinylaiton in the cells co-transfected GluR1/GluR2 with p97 or vector as a control. We found that co-expression with p97 significantly reduced cell surface expression level of GluR1 (p97 0.8082 ± 0.02206 normalized to vector, n = 4, p = 0.003200 vs. vector; Fig. 3.8D), but not GluR2 (p97 0.9735 ± 0.6037 normalized to
Figure 3.8 p97 depresses the cell surface expression of homomeric GluR1 receptors in the GluR1/GluR2 cotransfected HEK 293 cells

(A) Sample traces of the currents induced by 50 μM kainic acid while holding the cells at -60, -40, -20, 0, 20, 40, 60 mV in the HEK 293 cells expressing GluR2; GluR1, GluR2 and p97; GluR1, GluR2 and vector; or GluR1.

(B) I-V curve summarized from 6-8 cells of each group shown in (A). The amplitudes of the currents at different holding potentials were normalized to the current at -60 mV. I-V curve was left shifted to GluR2 alone when GluR1 and GluR2 were co-transfected with p97 compared with vector.

(C) Quantification of rectification index (I_{60/-60}) of each group shown in (A). Co-expression of p97 increased the rectification index of GuR1/GluR2 co-transfected group to a level comparable to GluR2 alone. * p < 0.05, ** p < 0.01, ANOVA followed by post hoc tests.

(D) The composition changes of the surface expressed AMPARs are due to decreased surface expression of GluR1 by p97. Surface proteins of the cells co-transfected with HA tagged GluR1, none-tagged GluR2, and p97 or vector as control were labeled by biotinylation. GluR1 or GluR2 in the 1/5 of the amount of lysates used in avidin pull down were immunoprecipitated by anti-HA (for GluR1) or anti-GluR2 antibody, respectively, to control the total expression. p97 significantly decreased surface expression of GluR1 but not GluR2, as shown in the representative western blot images (left) and the quantification (right) of surface GluR1 or GluR2 from four individual experiments (surface intensity of GluR1 or GluR2 was normalized to total, and then
normalized to control (vector)). * p < 0.05 Student’s t-test compared with control (vector).
vector, n = 4, p = 0.6901 vs. vector; Fig. 3.8D). Also, p97 did not change the total expression level of GluR1 or GluR2 (Fig. 3.8D). In summary, these results suggest that although p97 facilitates the formation of homomeric GluR1 AMPARs, association with p97 keeps these receptors in a reserved intracellular pool.

### 3.3.6 p97 keeps homomeric GluR1 AMPARs in the intracellular pool in cultured hippocampal neurons

The presence of such a reserved pool of GluR2-lacking, homomeric GluR1 AMPARs has been identified in hippocampus neurons and would be expected to have fundamental physiological and pathological significance since the channel in the GluR2-lacking AMPAR, in comparison with that of GluR2-containing receptor, has a much higher calcium permeability (Cull-Candy et al., 2006; Isaac et al., 2007). In order to study whether p97 also affects the sub-cellular location of homomeric GluR1 receptors in hippocampal neurons, we transfected cultured neurons with GluR1 and p97, as well as vector as control, 14 days after plating. Miniature excitatory postsynaptic currents (mEPSCs) were recorded under whole-cell configuration 2-3 days post-transfection. Furthermore, the synaptic homomeric GluR1 receptors were measured by changes in the amplitude and frequency of mEPSCs following the application of a GluR2-lacking AMPA antagonist philanthotoxin-433 (PhTx, 10 μM). Previous work using the transfected GluR1 in cultured hippocampal neurons to study the trafficking of GluR1 showed that the exogenous GluR1 only forms homomeric receptors, and cannot combine with endogenous GluR2 to form heteromeric receptors (Hayashi et al., 2000). In addition, these homomeric GluR1 receptors can be inserted into the cell surface under basal
conditions as evidenced by the increased inward rectification of I-V relationship of AMPAR current responses (Shi et al., 1999). Consistent with these previous work, we found that in the neurons transfected with GluR1 and vector, PhTx significantly decreased the frequency and amplitude of mEPSC (Frequency: 27.28 ± 5.773 %, n = 6, p = 0.001720 vs. basal; Amplitude: 83.84 ± 3.995 %, n = 6, p = 0.009587 vs. basal; Fig. 3.9 A, B and C), which suggests that exogenous GluR1 formed homomeric GluR1 AMPARs and these receptors can be inserted into the postsynaptic membrane. However, in neurons transfected with GluR1 and p97, the effects of PhTx in the reduction of frequency and amplitude of mEPSC is much weaker (Frequency: 85.32 ± 5.318 %, n = 5, p = 0.1014 vs. basal; Amplitude: 96.13 ± 0.5037 %, n = 5, p = 0.009742 vs. basal; Fig. 3.9 A, B and C), suggesting less homomeric GluR1 AMPARs were present on the postsynapses when they are associated with p97. The big difference of synaptic level of homomeric GluR1 AMPARs between with and without coexpression of p97 (Change in frequency: -72.72 ± 5.773 %, n = 6 for GluR1 + Vector, p = 0.00004150 vs. -14.68 ± 5.318 %, n = 5 for GluR1 + p97; Change in amplitude: -16.16 ± 3.995 %, n = 6 for GluR1 + Vetor, p = 0.02719 vs. -3.867 ± 0.5037 %, n = 5 for GluR1 + p97; Fig. 3.9C) suggests p97 is important in keeping homomeric GluR1 AMPARs in the intracellular reserved pool in hippocampal neurons.

3.3.7 LTP, but not LTD induces dissociation of p97-GluR1 interaction

p97-GluR1 interaction restricts the homomeric GluR1 receptors in the intracellular pool, which is consistent with the lack of homomeric GluR1 receptors in the cell surface under the basal conditions (Plant et al., 2006; Toth et al., 2000). However, rapid regulation of AMPARs has been implicated in the expression and maintenance of
Figure 3.9 p97 inhibits the postsynaptic surface expression of homomeric GluR1 receptors in hippocampal neurons

Homomeric GluR1 receptor mediated mEPSC was measured by application of a GluR2-lacking AMPAR antagonist PhTx (10 μM) in the cultured hippocampal neurons transfected with GluR1 + p97, or GluR1+ vector as control.

(A) Examples of continuous recordings from an individual neuron of each group before (basal) and after PhTx application.

(B) Cumulative probability plot for mEPSC amplitudes and interevent intervals obtained before (basal) and after PhTx application shown in (A).

(C) Quantification of the changes of mEPSC frequency and amplitude by application of PhTx from 6-7 individual neurons for each group. The frequency and amplitude of mEPSC recorded after PhTx application were compared with mEPSC before PhTx. In addition, the changes in frequency and amplitude represent the expression level of homomeric GluR1 receptors on the postsynaptic membrane. The decreased sensitivity to PhTx in neurons expressing GluR1+ p97 compared with GluR1+ vector indicated that p97 reduces the synaptic surface expression of homomeric GluR1 receptors. * p < 0.05, ** p < 0.01, Student’s t-test compared with control (vector).
synaptic plasticity, including LTP and long-term depression (LTD), the two most well-studied forms of synaptic plasticity (Malenka and Bear 2004). In order to study whether the p97-GluR1 interaction is affected by LTP or LTD, we used well-characterized chemical LTP and LTD models in cultured hippocampal neurons. Bath application of glycine (200 μM; 3 min) or NMDA (10 μM; 3 min) can induce LTP or LTD in cultured hippocampal neurons, respectively (Beattie et al., 2000; Lu et al., 2001b). Ten minutes after drug application, cell lysates were collected for co-immunoprecipitation of p97-GluR1 complex using anti-GluR1 antibody. The interaction of p97 with GluR1 was dramatically decreased during LTP (0.5881 ± 0.09927 normalized to control, n = 4, p = 0.01903 vs. control; Fig. 3.10A), but did not change during LTD (0.9824 ± 0.1483 normalized to control, n = 4, p = 0.9410 vs. control; Fig. 3.10A). The specific dissociation of p97-GluR1 complex suggests that the release from p97 may modulate the trafficking of homomeric GluR1 receptors during LTP.

Transient insertion of homomeric GluR1 AMPARs at synapses shortly after LTP induction was previously shown to play a crucial role for the full expression of LTP (Gray et al., 2007; Isaac et al., 2007; Plant et al., 2006). However, the mechanisms by which LTP induction rapidly alter the composition of AMPAR, and thereby increases in the insertion of GluR1 homomeric AMPARs into synapses remain unknown. Given our results that p97 specifically interacts with homomeric GluR1 AMPARs and keeps a reserved pool of this subtype of receptors under the basal conditions; and that p97 dissociates from homomeric GluR1 receptors by a LTP-like stimulation in hippocampal neurons, we hypothesize that p97, by increasing and keeping an intraocular pool of GluR1 homomeric AMPARs and releasing them from the intracellular pool, may play a
Figure 3.10 Dissociation of p97-GluR1 after LTP induction in cultured hippocampal neurons

(A) LTP and LTD were induced by 3 min 200 μM glycine and 3 min 10 μM NMDA, respectively, as previously described (Man et al., 1999). Cell lysates collected before (as control) and 10 min after treatment were co-immunoprecipitated with anti-GluR1 antibody, followed by sequentially probing with anti-p97 and anti-GluR1 antibodies. p97-GluR1 interaction was significantly decreased in LTP, but not LTD as shown in the representative Western Blot images (top) and the quantification (bottom) of p97 co-immunoprecipitated by GluR1 from four individual experiments (intensity of p97 was normalized to GluR1, and then normalized to control). * p < 0.05, Student’s t-test compared with control.

(B) Dissociation of p97-GluR1 complex shortly after glycine stimulation. Cell lysates collected before (as control) and at different time points (5 min, 30 min, and 60 min) after glycine stimulation were co-immunoprecipitated as described in (A). p97-GluR1 interaction was significantly decreased at 5 min and recovered to the normal level 60 min after LTP induction, as shown in the representative Western Blot images (top) and the quantification (bottom) of p97 co-immunoprecipitated by GluR1 from 6 individual experiments (intensity of p97 was normalized to GluR1, and then normalized to control). * p < 0.05, Student’s t-test compared with control.
critical role in the expression of early phase of LTP. In order to test the time course of p97-GluR1 dissociation, we carried out co-IPs at the different time points after glycine stimulation, found a significant decrease of p97-GluR1 interaction 5 min after LTP induction (0.5523 ± 0.1016 normalized to control, n = 8, p = 0.02145 vs. control; Fig. 3.10B). In addition, the interaction of p97 with GluR1 returned to a normal level 60 min after LTP induction (30 min 0.8142 ± 0.1442 normalized to control, n = 7, p = 0.1348 vs. control; 60 min 1.025 ± 0.1480, n = 6, p = 0.8936 vs. control, Fig. 3.10B). The dissociation of p97-GluR1 at 5 min after LTP induction could provide a mechanism for the synaptic insertion of homomeric GluR1 receptors shortly after LTP induction previously observed in slices (Plant et al., 2006).

3.3.8 Insertion of homomeric GluR1 AMPARS into postsynapses shortly after glycine stimulation in cultured hippocampal neurons

To test whether the transient insertion of homomeric GluR1 AMPARs at synapses shortly after LTP also occurs in cultured hippocampal neurons, we utilized a glycine induced LTP model in cultured hippocampal neurons (Lu et al., 2001b). Five min after glycine stimulation, we found a potentiation of frequency and amplitude of mEPSC (Frequency: 264.1 ± 31.93 %, n = 7, p = 0.002481 vs. baseline; Amplitude: 106.1 ± 2.000 %, n = 7, p = 0.03105 vs. baseline; Fig. 3.11 A, B, and C). Similar to the change of subunit composition of AMPARs shortly after LTP induction in CA1 neurons of hippocampal slices (Plant et al., 2006), both frequency and amplitude of mEPSCs were significantly decreased by application of GluR2-lacking AMPAR specific inhibitor PhTx
Figure 3.11 Synaptic insertion of homomeric GluR1 receptors after LTP induction in cultured hippocampal neurons

Homomeric GluR1 receptor mediated mEPSC was measured by application of a GluR2-lacking AMPAR antagonist PhTx (10 μM) in the cultured hippocampal neurons incubated in extracellular solution (ECS; control), or 5 min after glycine (200 μM, 3 min; LTP) application.

(A) Examples of continuous recordings from an individual control neuron before (basal), and after PhTx application; and an individual LTP neuron before (basal), after glycine application, and after PhTx applied 5 min after glycine.

(B) Cummulative probability plot for mEPSC amplitudes and interevent intervals obtained before (basal), and after PhTx application for control; and before (basal), after glycine application, and after PhTx applied 5 min after glycine for LTP.

(C) Quantification of the changes in mEPSC frequency and amplitude by application of PhTx from 6-7 individual neurons for each group. The frequency and amplitude of mEPSC recorded after PhTx application were compared with mEPSC before PhTx. Moreover, the changes in frequency and amplitude represent the expression level of homomeric GluR1 receptors on the postsynaptic membrane. The higher sensitivity to PhTx 5 min after glycine stimulation indicates the synaptic insertion of homomeric GluR1 receptors. * p < 0.05, ** p < 0.01, Student’s t-test compared with control.
(10 μM) 5 min after glycine stimulation (Frequency: 52.30 ± 5.021 %, n = 7, p = 0.002332 vs. 5 min after glycine; Amplitude: 90.93 ± 1.906 %, n = 7, p = 0.001908 vs. 5 min after glycine; Fig. 3.11 A, B, and C). In contrary, application of PhTx produced little effect on the basal mEPSCs in non-glycine (non-LTPed) treated neuronal cultures (Frequency: 99.68 ± 5.953 %, n = 6 vs. baseline; Amplitude: 97.64 ± 0.8170 %, n = 6 vs. baseline; Fig. 3.11 A, B, and C), consistent with the absence of homomeric GluR1 receptors at synapses under basal conditions shown in other studies (Plant et al., 2006; Toth et al., 2000). The significant decrease of frequency and amplitude of mEPSC 5 min after glycine compared with the basal mEPSC (change in frequency after PhTx: -47.70 ± 5.021 %, n = 7 for LTP, p = 0.0001044, vs. -0.3217 ± 5.953 %, n = 6 for control; change in amplitude after PhTx: -9.075 ± 1.906 %, n = 7, p = 0.01172 vs. -3.258 ± 0.8170 %, n = 6 for control; Fig. 3.11C), strongly suggests that a rapid increase in the synaptic insertion of GluR1 homomeric AMPARs takes place shortly after LTP induction in cultured hippocampal neurons in a similar process observed in CA1 neurons of hippocampal slices (Plant et al., 2006).

### 3.3.9 Overexpression of p97 abolished LTP induced by glycine in cultured hippocampal neurons

One way to ascertain the role of a protein in a biological function is to delete the protein and assay function in its absence. However, studies which tried to knockout p97 in mice found that p97 gene ablation leads to early embryonic lethality (Muller et al., 2007). Also, RNA interference of p97 reduces proliferation of HeLa cells and causes accumulation of polyubiquitinated proteins (Wojcik et al., 2004), which affects the
normal cell activities. In our preliminary experiments (data not shown), we found that overexpressing a dominant negative mutant, p97QQ, was very toxic to neurons, which is not surprising given the numerous functions of p97 in many cellular processes (Wang et al., 2004). Therefore, in the present study, rather than knocking down p97, we overexpressed wild type p97 as a positive modulator to increase the 97-GluR1 interaction. Thus, cultured hippocampal neurons were transfected with p97 or vector as a control, 14 days after plating. Forty-eight to seventy-two hours after transfection, mEPSC was recorded in the presence of GABA\textsubscript{A}R and voltage-gated sodium channel blockers. A 3 min glycine (200 μM) treatment induced a long-lasting potentiation of mEPSC (Change in frequency 20 min after glycine: 69.2 ± 23.60 %, n = 7, p = 0.01678 vs. baseline; Fig. 3.12 A and B) in the control neurons as reported previously (Lu et al., 2001; Man et al., 2003). However, in neurons overexpressing p97, glycine stimulation failed to induce LTP (Change in frequency 20 min after glycine: -17.42 ± 23.60 %, n = 6, p = 0.2566 vs. baseline; Fig. 3.12 A and B).

The blockade of LTP by overexpression of p97 could be caused by abnormal modulation of homomeric GluR1 AMPARs after glycine stimulation. Considering that association of p97 retains homomeric GluR1 AMPARs intracellularly, excessive interaction with p97 following overexpression of p97 may inhibit the activity-dependent release of p97 from the homomeric GluR1 AMPARs and consequently reduce the insertion of these receptors into synapses following LTP.
Figure 3.12 Overexpression of p97 abolished glycine induced LTP in cultured hippocampal neurons

Cultured hippocampal neurons were transfected with p97 or vector as control, and mEPSC was recorded 48-72 hours after transfection.

(A) Examples of continuous recordings from individual neurons immediately before (basal) and 20 min after glycine (200 μM, 3 min) application.

(B) Quantification of frequency of mEPSCs from 6-7 individual neurons in each group. Frequency of the responses obtained 20 min after glycine application was normalized to basal responses. LTP could not be induced by glycine in the neurons overexpressing p97 compared with vector. * p < 0.05 student t-test.
3.4 Discussion

In this study, we identified a novel AMPAR associated protein p97. p97, a highly abundant chaperon protein that is found in various cell types, has been shown to play a crucial role in several cellular activities (Dreveny et al., 2004; Wang et al., 2004). Our data presented here firstly demonstrates that p97 is specifically associated with homomeric GluR1 AMPARs, and modulates their trafficking in cultured hippocampal neurons. The p97-GluR1 association is a direct protein-protein interaction, which depends on multiple domains within the N-terminal of GluR1. The direct binding to N-terminal of GluR1, but not GluR2 allows p97 to reduce the formation of GluR1/GluR2 heteromeric receptors, thereby promoting the formation of homomeric GluR1 receptors. The association with p97 decreases the surface expression of homomeric GluR1 receptors in both the transfected cell lines and cultured hippocampal neurons. This is consistent with the location of homomeric GluR1 receptors in an intracellular reserved pool of the hippocampal neurons under basal conditions as previously reported (Plant et al., 2006; Toth et al., 2000). Similar to earlier findings from LTP in hippocampal slices (Plant et al., 2006), we found a significant synaptic insertion of homomeric GluR1 receptors shortly after glycine induced LTP of mEPSC in cultured hippocampal neurons, which is correlated with a rapid dissociation of GluR1-p97 complex. Moreover, overexpression of p97, which increases the restriction strength and inhibits the insertion of homomeric GluR1 receptors into synapses, blocks LTP. Taken together, our results strongly suggest that by regulating the formation and trafficking of homomeric GluR1 AMPARs, p97 may be a critical molecule that is directly involved in mediating the expression of early phase of LTP.
3.4.1 Difference between p97 and NSF

As previously mentioned, p97 is closely related to NSF, both of which belong to type II AAA ATPase family. Interestingly, NSF has been shown to specifically bind to GluR2 and is involved in plasma membrane insertion and/or stabilization of GluR2-containing AMPARs (Collingridge et al., 2004). Similar to NSF, p97 is a multifunctional enzyme that is involved in protein trafficking related membrane fusion events (Uchiyama and Kondo, 2005; Vedrenne and Hauri, 2006). Thus, the specificity of p97 as a unique GluR1-binding protein makes it an ideal candidate to regulate GluR1-dependent AMPAR trafficking. However, different from the NSF-GluR2 interaction, which is dependent on a 10 amino acid sequence (844–853) within the C-terminal of GluR2 (Nishimune et al., 1998), the p97-GluR1 interaction involves multiple domains within the N-terminal of GluR1, making it almost impossible to develop a short interfering peptide. The NSF-GluR2 interaction is proposed to play a role in the stabilization of surface AMPA receptors on the postsynaptic membrane since a peptide that disrupts the NSF-GluR2 interaction causes a rapid decrease in synaptic currents (Kim and Lisman, 2001). In contrast, p97-GluR1 interaction depresses the surface expression of homomeric GluR1 AMPARs, and disruption of this interaction promotes the receptor’s postsynaptic insertion. Thus, the selectivity of NSF and p97 in interacting with different subtypes of AMPARs (GluR2-containing and GluR2-lacking) makes them play distinct roles in the modulation of AMPAR trafficking at different conditions.

3.4.2 p97 is a novel AMPAR N-terminal associated protein

Several studies have focused on the proteins that interact with the intracellular, C-terminal domains of the GluR subunits that link receptor function to cytoskeletal
elements and signaling molecules. These interactions play a critical role in synaptic plasticity and therefore, have been the interest of much research. However, recent work demonstrates a role for AMPAR N-terminal protein-protein interactions either in receptor trafficking or in synaptogenesis. Neuronal-activity-regulated pentraxin (Narp), a secreted neuronal pentraxin, has been identified to interact with N-terminal of AMPA receptors, and promotes the clustering of AMPARs (O'Brien et al., 1999; Sia et al., 2007).

Moreover, the cell adhesion molecule N-cadherin directly interacts with the extracellular N-terminal domain of GluR2, and has been shown to be involved in promoting the formation and growth of dendritic spines in cultured hippocampal neurons (Saglietti et al., 2007). Since the extracellular N-terminal is the longest domain among the entire structure of GluR subunits, it is not surprising that other unknown interacting protein partners may modulate AMPARs through the interaction with their N-terminals. In this study, we discovered that p97 specifically interacts with GluR1 homomeric AMPA receptors and modulates receptor trafficking. In addition, association with p97 affects the subunit composition of AMPARs. The selectivity of p97 to bind N-terminal of GluR1, but not GluR2 facilitates the formation of homomeric GluR1 receptors.

Considering the fact that multiple subtypes of AMPARs are expressed in the hippocampus, and distributed differently throughout the brain, it is interesting to study whether the expression level of p97 also follows this pattern of change in the level of homomeric GluR1 receptors. Using focal and global ischemia animal models, we have found a significant increase of p97-GluR1 interaction in areas of the brain subjected to ischemia (data not shown) as well as a slight increase in the expression level of p97. This may at least in part explain the selective alteration in the AMPAR subunit compositions.
after ischemic brain insults in CA1 of hippocampus (Oguro et al., 1999; Pellegrini-Giampietro et al., 1992). Thus, the change of GluR1-p97 interaction, which reflects the change of expression of homomeric GluR1 receptors, may provide a new tool to study the function of homomeric GluR1 receptors in physiological and pathological conditions.

3.4.3 p97 facilitates homomeric GluR1 AMPAR formation

AMPAR subunits are synthesized and assembled as dimers of dimers (Ayalon et al., 2005; Ayalon and Stern-Bach, 2001; Tichelaar et al., 2004) in the rough endoplasmic reticulum (ER) and then inserted into the plasma membrane after crossing the Golgi apparatus. The N-terminal domain, which is co-translationally inserted into the rough ER through the Sec61 channel (Clemons et al., 2004), mediates the initial step of dimer formation, and subsequent tetramerisation involves the extracellular S2 loop and the transmembrane segments (including the pore loop) (Ayalon and Stern-Bach, 2001). Dimerization of AMPAR subunits have been shown to prefer to form heteromers rather than simply homodimerize (Mansour et al., 2001). How this first assembly step is achieved is unclear, but it mainly due to the tight interaction between N-terminals of different subunits (Kuusinen et al., 1999). Although the majority of AMPARs in hippocampus is GluR1/GluR2, GluR2/GluR3 heteromeric receptors, homomeric GluR1 receptors are still present and account for about 8% of total AMPARs (Wenthold et al., 1996). The specific interaction of p97 with GluR1 provides us a mechanism why the non-preferred GluR1 homomers can exist and play an important role in several physiological and pathological conditions. Actually, selectivity of p97 to interact with the N-terminal of GluR1 but not GluR2 significantly decreases the formation of heteromeric receptors in heterogenous cells (Fig. 3.7). Considering the time course of
AMPAR subunits dimerization, the effects of p97 on the heteromeric receptor formation must take place at an early stage of subunits synthesis. We predict that the p97-GluR1NT interaction may happen co-translationally in the rough ER, and association of p97 with GluR1 allows the insertion of p97 into ER lumen accompanying GluR1. However, the obstacle between p97 and GluR2 forbids the original preferred GluR1-GluR2 dimerization, thus facilitating the GluR1 homodimerization. The co-translational GluR1-p97 interaction has not been directly proven, however, several pieces of evidence has provided strong support. GluR1 only interacts with co-expressed exogenous p97, but not endogenous p97 in COS-7 cells (Fig. 3.5A), which is likely because exogenous p97 can be expressed simultaneously and in the same compartment as GluR1. In addition, the p97-GluR1NT interaction decreases the formation of GluR1-GluR2 heteromers, a process that takes place co-translationally at the early biosynthesis stage of each subunit in the ER. Taken together, p97 affects AMPAR subunit composition, which is probably through the co-translational interaction with GluR1NT in the ER.

3.4.4 p97 modulates homomeric GluR1 AMPAR trafficking

The association with p97 not only increases the formation of the homomeric GluR1 receptors, but also keeps them in the intracellular reserved pool under the basal conditions (Fig. 3.9). Availability of such a pool of GluR2-lacking, homomeric GluR1 AMPARs would be expected to have fundamental physiological and pathological significance (Cull-Candy et al., 2006; Isaac et al., 2007). Stimulated insertion of homomeric GluR1 AMPARs has been demonstrated under many physiological and pathological conditions (Cull-Candy et al., 2006; Isaac et al., 2007; Plant et al., 2006). However, the underlying mechanisms remain unknown. Using the experimental
approaches combining surface receptor biotinylation, surface receptor immunofluorescence imaging and electrophysiological recordings, we documented that p97 dramatically decreases the cell surface expression of GluR1, but not GluR2, AMPARs (Fig. 3.5 A, B, and C), resulting in an almost exclusive expression of GluR2-containing AMPARs on the plasma membrane in the heterogeneous expression system (Fig. 3.8 A and B). In hippocampal neurons, co-expression of p97 causes most of the exogenously expressed homomeric GluR1 receptors in the intracellular sites (Fig. 3.9), which mimic the status of endogenous homomeric GluR1 receptors.

The rapid activity-dependent changes in the composition of AMPA receptors have been shown to mediate several forms of synaptic plasticity. Homeostatic synaptic plasticity, which is induced by chronically blocking neuronal activity or glutamatergic transmission in cultured neurons, involves in the composition switch from GluR2-containing to GluR1-dominated synaptic AMPA receptors (Thiagarajan et al., 2005). An increase in GluR1, but not GluR2, protein expression is observed in response to activity blockade (Ju et al., 2004; Sutton et al., 2006). Recent work by Isaac and co-workers showed that activity-dependent alteration in the composition of AMPA receptor also occur at the Schaffer collateral-CA1 synapse, which expresses primarily Ca\(^{2+}\)-impermeable AMPA receptors (Plant et al., 2006), and the transient presence of the GluR2-lacking AMPA receptors plays a crucial role in full expression of LTP, because blockade of GluR2-lacking AMPA receptors during the early phase of expression causes a reversal of LTP. In this study, we used a well-studied chemical LTP model in cultured hippocampal neurons (3 min 200 μM glycine), which shares similar mechanisms with hippocampal slices (Lu et al., 2001b; Man et al., 2003). We found a significant insertion
of homomeric GluR1 receptor into synapses 5 min after LTP induction, which was reflected by the decrease in frequency and amplitude of mEPSC after the application of a GluR2-lacking AMPAR antagonist PhTx. Furthermore, the trafficking of homomeric GluR1 receptors from the intracellular reserved pool to postsynaptic membrane is accompanied with the dissociation of p97-GluR1 interaction. Losing of the restricting strength from p97 may be involved in the mechanism of the change of composition of AMPARs shortly after LTP induction. The dissociation of p97-GluR1 complex only occurs by a stimulus that induces LTP, but not LTD (Fig. 3.10), and overexpression of p97, which increases the restriction strength of keeping homomeric GluR1 receptors in the intracellular site, blocks the expression of LTP (Fig.3.11). Thus, the modulation of homomeric GluR1 receptor trafficking by p97 highlights a new area to study the mechanism of LTP. However, how p97 dissociates from homomeric GluR1 receptors remains unknown. The modulation of p97 activity has not been well studied, but several lines of evidence have shown the involvement of oxidative stress (Noguchi et al., 2005), and phosphorylation (Klein et al., 2005; Vandermoere et al., 2006). In addition, LTP induction activates several protein kinases which phosphorylate GluR1, such as Ser 831 by PKC and CaMKII and Ser 845 by PKA, which have been shown to play an important role in the expression of LTP (Hayashi et al., 2000; Malinow, 2003). Any confirmation change of either p97 or GluR1 by the glycine stimulation may initiate the dissociation. The identification of the molecules downstream of the activation of NMDAR which modulate either p97 or GluR1 should facilitate our efforts in understanding the molecular mechanisms of LTP.
3.5 Supplementary materials

3.5.1 Methods for plasmid construction

R1Δ809-889 was generated using a Quick-Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with primers TTGCCTTAATCTAGTTCTGCTAC and GTAGCAGAACTAGATTAAGGCAA to mutate GluR1 809 GAG to TAG.

R1Δ594-889 was generated using a Quick-Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with primers CAGGTCCCTGTCTAGGCATCGTC and GACGATGCGGCAGGACGGGACCTG to mutate GluR1 594 GGA to TGA.

R1Δ542-889 was generated using a Quick-Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with primers TCCTGTCAGCCGTGTTGAAGGCCCTAGGAATGG and CCATTCGTAGGGGTCTCAACGGCTGACCAGGA to mutate GluR1 542 TTC to TGA. R1Δ510-541 was generated using a Quick-Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with primers CAGAAGTCCAAGCCAGGTTGCTCAGCCCTACGAATGG and CCATTCGTAGGGGTCTCAACGGCTGACCAGGA to mutate GluR1 542 TTC to TGA. R1Δ510-541 was generated using a Quick-Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with primers CAGAAGTCCAAGCCAGGTTGCTCAGCCCTACGAATGG and CCATTCGTAGGGGTCTCAACGGCTGACCAGGA to mutate GluR1 542 TTC to TGA.

For switching N-terminal of GluR1 with N-terminal of GluR2, HindIII site in the N-terminal of HA-GluR1 was generated by point mutation immediately before the first transmembrane domain using the primers CATTATGATTAAGCTTCAGAAGTCCAAGCCAGG and CCATTCGTAGGGGTCTCAACGGCTGACCAGGA to delete GluR1 510-541.

For switching N-terminal of GluR1 with N-terminal of GluR2, HindIII site in the N-terminal of HA-GluR1 was generated by point mutation immediately before the first transmembrane domain using the primers CATTATGATTAAGCTTCAGAAGTCCAAGCCAGG and CCATTCGTAGGGGTCTCAACGGCTGACCAGGA to delete GluR1 510-541.

HindIII site in the N-terminal of HA-GluR2 was generated by point mutation immediately before the first transmembrane domain using the primers CATTATGATTAAGCTTCAGAAGTCCAAGCCAGG and CCATTCGTAGGGGTCTCAACGGCTGACCAGGA to delete GluR1 510-541.
TATCATGATCAAGAAGCCTTCAGAAGTCCAAACCAGG and
CCTGGTTTTGACTTCTGAAGCCTTCTTGATCATGATA to mutate 528 CCT to CTT.
After digestion by HindIII, the resulting backbone of HA-GluR1 was ligated to the
GluR2 N-terminal. GST-R1N and GST-R2N were generated by PCR amplification of
GluR1 NT (4-503) using primers CCGGAATTCCCCAACAATATCCAGATAGGG and
TGCGGTGACTTATGGCTTCTTTAATCATAAT, and GluR2 NT (1-528) using
primers CCGGAATTCATGCAAAAGATTATGCATATT and
TGCGGTGACTTAAGGCTTCTTGATCATGATAGAG, and inserted into EcoRI and
Sall sites of pGEX 4T-1 vector (Amersham, Piscataway, NJ, USA). GluR1 4-374 was
generated by PCR amplification using primers
CCGGAATTCCCCAACAATATCCAGATAGGG and
TGCGGTGACTTATGGCTTCTTTAATCATAAT; 376-503 using primers
GCCCGAATCGGGGACGCTCAGGCTGGAGGGGAC and
TGCGGTGACTTATGGCTTCTTTAATCATAAT; 4-120 using primers
CCGGAATTCCCCAACAATATCCAGATAGGG and
TGCGGTGACTTAGTCGATAATGCTAATGAGAGC; 121-240 using primers
GCCCGAATTCGGCATTACAAGTGCGGAAACCTTT and
TGCGGTGACTTAGTAGTTCACCAGCTGGAAACC; 241-374 using primers
GCCCGAATTCGGACAGACACGATCCAGCC and
TGCGGTGACTTAGGCTGCGGGGACAAA; 416-503 using primers
GCCCGAATTCGGCGCTATGAGGGCTACTGTGTG and
TGCGGTGACTTATGGCTTCTTTAATCATAAT; 376-456 using primers
GCCCGAATCGGGGACGCTCAGGCTGGAGGGGAC and
TGCGGTCGACTTACCAAGCCTTTTGTCGGG; 456-503 using primers GCCCGAATTGCCGTAATGGCATGGTGAGGAGA and TGCGGTCGACTTATGGCTTCTTAATCATAAT, and inserted into EcoRI and SalI sites of pCMV-HA vector (Clontech, Mountain View, CA, USA). R1Δ121-240 was generated using a Quick-Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with primers CTCATTAGCATTATCGACACAGACACGATCCCAGCC and GGCTGGGATCGTGTCTGTGTCGATAATGCTAATGAG to delete GluR1 121-240.

R1Δ416-503 was generated using a Quick-Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with primers CAGTTTGAGGGCAATGACCCACACAGAAAGTCCAAGCCA and TGGCTTGGACTTCTGTGGGTCATTGCCCTCAAACTG to delete GluR1 416-503.

p97 expressed in mammalian system was generated by PCR amplification of p97 using primers TAATGAAAGCGGCCGCCAATGGCCTCTGGAGCCGAT and CGCGGATCCTTAGCCATACAGGTCATCGC, and inserted into NotI and BamHI sites of pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA).
3.6 References


4. CONCLUDING REMARKS

4.1 Research summary

Long-term synaptic plasticity including LTP and LTD, are believed to be the foundation for higher brain functions such as learning and memory (Bliss and Collingridge, 1993; Kemp and Manahan-Vaughan, 2007; Martin et al., 2000). The distinct roles of hippocampal LTP and LTD in the contribution of spatial memory formation remain unclear, even though traditionally LTP has been designated as the main mediator of spatial memory storage in the hippocampus, whereas LTD has been assigned an auxiliary role in creating a higher signal-to-noise ratio (Dayan and Willshaw, 1991) or in erasing stored information (Tsumoto, 1993). However, recent studies have shown that LTP and LTD have different roles in spatial information processing. Indeed, in vivo electrophysiological recording in freely moving rats showed that LTP and LTD are facilitated by exposing rats to empty space and novel objects, respectively (Kemp and Manahan-Vaughan, 2004, 2005; Lemon and Manahan-Vaughan, 2006; Manahan-Vaughan and Braunewell, 1999), suggesting that LTP and LTD encode different types of information during spatial learning.

In support of the hypothesis that hippocampal LTP and LTD play distinct roles in the spatial memory formation, the present work found: 1) In freely moving adult rats, i.p. injection of NR2A antagonist NVP selectively prevented LTP formation in a similar level as nonselective NMDAR antagonist CPP, but did not affect LTD formation in hippocampus. In addition, hippocampal LTD, which is inducible in freely moving adult rats, was abolished by either a NR2B antagonist Ro that inhibits LTD induction or Tat-GluR23Y peptide, a synthetic peptide that disrupts LTD expression through blocking
AMPAR endocytosis. Ro and Tat-GluR23Y peptide showed a similar effect as CPP in blocking the induction of LTD, and did not affect the induction of LTP. Given the selective blockade of LTP by NVP and LTD by Ro and GluR23Y under similar conditions where animals’ ability of learning and memory are tested, our results strongly indicate these reagents are very useful tools in probing specific roles of LTP and LTD in learning and memory in freely moving adult rats. 2) Preventing LTP formation in freely moving rats using NVP did not affect either the requisition or recall of Morris-water-maze (MWM). 3) Both Ro and Tat-GluR23Y peptide, which prevent LTD formation through different mechanisms, impaired the consolidation without affecting either requisition or recall of LSM formation. These findings strongly support the requirement of hippocampal LTD in LSM formation in genetically unaltered adult rats.

Using two structurally and mechanistically distinct LTD inhibitors, the present study provides convincing evidence for an essential role of facilitated GluR2-dependent AMPAR endocytosis and hence, the expression of LTD in the LSM. However, due to the challenged subunit specificity of NVP and the lack of another structurally and mechanistically different LTP inhibitor, we admitted that the foundation for our conclusion that hippocampal LTP may not have a critical role in the LSM remains shaky. This also highlights the importance and urgency in searching for a more specific LTP inhibitor, such as the GluR23Y peptide for LTD. Therefore, in the second part of this study, we investigated AMPAR associated proteins which modulate AMPAR trafficking during LTP. Our ultimate goal is to identify a new specific inhibitor that can selectively disrupt the GluR1-dependent insertion of AMPARs during LTP, i.e. the last step of the LTP expression.
Mass spectrometric analysis of the protein complex co-immunoprecipitated by an anti-GluR1 or anti-GluR2 antibody from hippocampal homogenates identified a novel AMPAR associated protein p97. p97, a highly abundant chaperon protein in various types of cells, has been shown to play a crucial role in several cellular activities (Dreveny et al., 2004; Wang et al., 2004). We found that the association of p97 has several effects on AMPARs: 1) p97 specifically interacts with GluR1, but not GluR2 subunit in heterogeneous expression system, and only interacts with homomeric GluR1 AMPARs in hippocampal neurons. 2) The p97-GluR1 association is a direct protein-protein interaction, which depends on multiple domains within the N-terminal of GluR1 subunit. 3) The direct binding to N-terminal of GluR1, but not GluR2 subunit allows p97 to depress the formation of GluR1/GluR2 heteromeric receptors, thus facilitating the formation of homomeric GluR1 receptors. 4) The association with p97 decreases the surface expression of homomeric GluR1 receptors in both the transfected cell lines and cultured hippocampal neurons, consistent with the location of homomeric GluR1 receptors in an intracellular reserved pool of hippocampal neurons under basal conditions as previously reported (Plant et al., 2006; Toth et al., 2000). 5) Homomeric GluR1 receptors are inserted into the postsynaptic membrane shortly after glycine induced LTP in cultured hippocampal neurons, which is accompanied with a dissociation of GluR1-p97 complex. 6) Overexpression of p97, which increases the restriction strength and inhibits the insertion of homomeric GluR1 receptors into postsynaptic membrane, blocks LTP. Taken together, our results strongly suggest the modulation of homomeric GluR1 AMPARs trafficking by p97, making it a critical molecule that is involved in mediating the early stage of LTP.
4.2 Discussion

The study presented here provides the first evidence in support of a requirement of hippocampal LTD in LSM formation in genetically normal adult rats. The previous work used transgenic mice that displayed deficits in either LTP (Abel et al., 1997; Kohr et al., 2003; Zamanillo et al., 1999) or LTD (Nicholls et al., 2008; Zeng et al., 2001) to test relative contributions of hippocampal LTP and LTD on LSM. The results obtained from these mice were hardly equivocal, with some transgenic mice exhibiting intact LSM (Bannerman et al., 2008; Nicholls et al., 2008; Reisel et al., 2002; Zamanillo et al., 1999; Zeng et al., 2001). Considering compensatory changes arising after prolonged genetic alterations (throughout brain development or for weeks in inducible knock-down studies), the transgenic mice are not good models for studying rapid changes in synaptic strength in LSM. In contrast, the LTP or LTD inhibitors used in this study, which prevent activation of corresponding subtypes of NMDARs or the endocytosis of AMPARs, acutely block the synaptic plasticity induced by the behavior tasks. Therefore, the roles of different forms of hippocampal plasticities in LSM can be revealed using these specific inhibitors. The requirement of hippocampal LTD in LSM suggests that in addition to previously believed as erasing stored information (Tsumoto, 1993) or creating a higher signal-to-noise ratio (Dayan and Willshaw, 1991), LTD directly mediates the formation of LSM. Furthermore, the specific inhibitors we used to manipulate different forms of hippocampal plasticities provide important tools for further dissecting the contribution of LTP and LTD in other hippocampal functions.

Moreover, our findings strongly suggest that hippocampal LTD is required for the rapid consolidation of spatial memory. The impairment of LSM by pre-training injection
of LTD inhibitors is likely due to the effects of drugs on the acquisition and consolidation of spatial memory. Since the acquisition of spatial memory is normal after pre-training injection of LTD inhibitors, the effects on the consolidation seems more responsible for the impairment of LSM. In addition, the post-training intra-hippocampal injection of Tat-GluR23Y peptide abolished LSM formation. Therefore, hippocampal LTD is required for the consolidation of LSM, and preventing LTD during the consolidation abolishes the formation of LSM.

In order to decrease the overlap of the acquisition and consolidation, the training protocol used in this study consisted of a single session of eight continuous trials. The traditional Morris water maze has multiple training trials lasting several days, and is not an ideal paradigm for dissociating acquisition and consolidation. The experiments based on this traditional paradigm found that the application of NMDAR antagonists 1 day (Morris, 1989; Morris et al., 1990) or 4 days (Heale and Harley, 1990) after the last day of training had no effect on the LSM. However, another study using the same paradigm as our study found that injection of AP5 or MK801 into rat hippocampus immediately after training on the MWM task impaired LSM when they were tested 24 h later (Packard and Teather, 1997a, b). Consistent with the study using a single session training protocol, our study found the intrahippocampal injection of Tat-GluR23Y peptide immediately after training abolished LSM formation. Compared with Ro, which inhibits the activation of NR2B-containing NMDARs, Tat-GluR23Y peptide blocks activity dependent endocytosis of AMPARs, the final expression stage of LTD. Since early consolidation could occur during the 8-trial training, direct delivery of the Tat-GluR23Y peptide into hippocampus is the most efficient way to block LTD expression in the early consolidation. Therefore,
our findings not only provide the first evidence that hippocampal LTD is required for the formation of LSM, but also suggest that the effects of LTD occur in the early stage of consolidation.

Activity-dependent changes in the subunit composition of AMPARs on the postsynapses mediate several forms of synaptic plasticity (Cull-Candy et al., 2006; Ju et al., 2004; Thiagarajan et al., 2005). Furthermore, transient synaptic incorporation of GluR2-lacking (possibly GluR1 homomeric) receptors after the induction of LTP is a necessary step for the full expression of LTP in the Schaffer collateral-CA1 synapses (Plant et al., 2006). The molecules that regulate the trafficking of the homomeric GluR1 receptors in hippocampal neurons remain unknown. In the second part of this study, we identified a novel AMPAR interacting protein, p97. Our data firstly demonstrate that p97 is specifically associated with homomeric GluR1 AMPARs, and by doing so, it modulates the formation and trafficking of homomeric GluR1 AMPARs in cultured hippocampal neurons. The specificity of p97-GluR1 interaction maintains an intracellular reserved pool of homomeric GluR1 receptors, which can be inserted into postsynaptic membrane upon the stimulus such as glycine which induces LTP in cultured hippocampal neurons. Thus, the modulation of homomeric GluR1 receptor trafficking by p97 provides a new molecular mechanism for LTP.

The interaction of p97 with GluR1 is dependent on the multiple domains within the N-terminal of GluR1, and can not be interrupted by a single domain deletion. Therefore, it is not easy to develop a short peptide to prevent the p97-GluR1 interaction. The original objective of this study is to identify new specific LTP inhibitors for studying the involvement of LTP in LSM. However, the nature of p97-GluR1 interaction makes it
different from the other single short interaction domain dependent protein-protein
interactions such as GluR2-NSF. Therefore, although the interaction of p97-GluR1 is
crucial for the expression of LTP in hippocampal neurons, developing a peptide-based
LTP inhibitor that targets on this protein-protein interaction is beyond the reach of this
study.

Although we could not identify the peptide-based LTP inhibitor, the investigation
of the GluR1-p97 interaction and the modulated trafficking of homomeric GluR1
receptors provide a new aspect for the study of expression mechanisms of LTP. It has
been believed that AMPARs are trafficking between the plasma membrane and
intracellular storages in a subunit specific manner (Hayashi et al., 2000; Passafaro et al.,
2001; Shi et al., 1999). The underlying mechanisms are not clear, but AMPAR subunit
phosphorylation/dephosphorylation and interaction with other proteins play an important
role in mediating subunit specific AMPAR trafficking (Santos et al., 2009). The specific
interaction of p97 with homomeric GluR1 receptors play a critical role in regulating the
trafficking of these receptors. Under the basal conditions, p97, via its association with
GluR1, retains homomeric GluR1 receptors in the intracellular sites. However, upon the
induction of LTP, such as following glycine stimulation, p97 rapidly dissociates from
homomeric GluR1 receptors. The dissociation of p97 then releases these receptors from
the intracellular pools and allows them to be inserted into the stimulated synapses,
thereby enabling the expression of LTP at these synapses.
4.3 Future directions

This work demonstrates that the requirement of LTD in LSM and modulation of homomeric GluR1 receptor formation and trafficking by a novel GluR1-associated protein p97. As detailed below, the present study in the meantime, also raised several important issues need to be further characterized in future work.

1. Can these specific LTP and LTD inhibitors be used to study roles of synaptic plasticity in other learning and memory behavioural tasks? Several lines of evidence have shown that synaptic plasticity is involved in several behavior tasks, such as hippocampus-dependent inhibitory avoidance conditioning (Impey et al., 1998; Taubenfeld et al., 1999; Whitlock et al., 2006) and fear conditioning which is dependent on the lateral amygdale (Blair et al., 2001; LeDoux, 2000; Maren et al., 2001; Rogan and LeDoux, 1995; Rogan et al., 1997). In this study, we demonstrated that hippocampal LTP can be selectively inhibited by NR2A antagonist NVP, whereas NR2B antagonist Ro and Tat-GluR23Y peptide specifically blocked the expression of LTD. Considering the synaptic plasticity shares a similar mechanism throughout the brain, these specific LTP and LTD inhibitors may also be functional in other brain regions. Thus, the roles of LTP and LTD in other behavior tasks could also be tested using the same inhibitors as we used in this study.

2. What are the signal pathways that modulate the dissociation of p97-GluR1 complex upon the stimulus that induces LTP? With the association of p97, homomeric GluR1 AMPARs are reserved in the intracellular site under the basal conditions. Application of glycine, which activates the synaptic NMDARs and induces the LTP of mEPSC (Lu et al., 2001b), dissociates p97 from GluR1 complex in culture hippocampal neurons. The
dissociation is accompanied with the synaptic insertion of homomeric GluR1 receptors, and this suggests that the dissociation of p97 may be a prerequisite step for the increase in homomemric GluR1 AMPAR insertion during LTP expression. Therefore, it is critically important to determine the mechanism underlying the dissociation of p97-GluR1 complex during the induction of LTP. Several protein kinases have been shown to be activated upon the activation of synaptic NMDARs, and both p97 and GluR1 are modulated by phosphorylation. Therefore, one line of research would be to determine whether some of the NMDAR-activated kinases and phosphatases can alter the levels of p97 and/or GluR1 phosphorylation, and thereby affect their association. Identification of these molecules downstream of the activation of NMDARs that regulate the association of p97 with GluR1, not only enriches our understandings of the molecular mechanisms of LTP, but also provides the potential targets for developing specific LTP inhibitors.

3. Does p97 co-traffic with homomeric GluR1 receptors? Since p97 binds to the N-terminal of GluR1, and affects the formation of homomeric GluR1 AMPARs, the p97-GluR1 complex is likely formed at the ER, during early stage of GluR1 synthesis processes. Then, important questions are raised as to whether p97 and GluR1 co-traffic together. Also, how does the dissociation of p97 affects homomeric GluR1 AMPAR insertion? As previously mentioned above, p97 may dissociate from GluR1 in the intracellular compartment just before GluR1 is being inserted. It is also plausible that some of p97 molecules are co-trafficked and inserted into extracellular plasma membrane with GluR1 as a p97-GluR1 complex following LTP induction. Once trafficked to the plasma membrane, p97 is then dissociated from the GluR1 receptor complex, allowing the receptor laterally diffused into synaptic membrane, contributing to the increased
synaptic homomeric GluR1 AMPARs. Indeed, previous studies do provide evidence supporting the presence of p97 in insoluble cortical membrane fractions (Zalk and Shoshan-Barmatz, 2003). If the cotrafficking of p97 and GluR1 to the plasma membrane surface is confirmed, it will then become important to determine whether some extracellular molecules released during LTP could regulate the p97-GluR1 interaction, causing the dissociation of p97 from the receptor and thereby triggering the lateral movement of the receptor into postsynaptic membrane. One potential extracellular modulator is ATP, which has been shown to be co-released with glutamate (Wieraszko et al., 1989; Zimmermann, 1994), directly binds to p97 and results in the changes in its confirmation and activity (Briggs et al., 2008; Davies et al., 2005; DeLaBarre and Brunger, 2005; Rouiller et al., 2000; Rouiller et al., 2002; Wang et al., 2003a). Moreover, in CA1 neurons, LTP can be induced by extracellular application of ATP (10 μM) during test electrical stimulation delivered at 0.05 Hz (Fujii et al., 1995).

4. Does p97 involve the modulation of homomeric GluR1 receptor trafficking?

Therefore, contributing alteration of AMPAR function under pathological conditions such as brain ischemia? In addition to synaptic plasticity, facilitated insertion of homomeric GluR1 AMPARs has also recently been implicated in some pathological brain disorders, including stroke (Liu et al., 2004b; Noh et al., 2005; Oguro et al., 1999). Ischemia insults cause selective, delayed neuronal death, primarily in hippocampal CA1 pyramidal neurons (Kirino, 1982; Pulsinelli et al., 1982), and a long-lasting switch in AMPA receptor composition, from GluR2-containing to GluR2-lacking receptors in vulnerable CA1 neurons (Calderone et al., 2003; Gorter et al., 1997; Liu et al., 2004b; Noh et al., 2005; Opitz et al., 2000; Pellegrini-Giampietro et al., 1997). Several lines of
evidence have shown that increasing the number of Ca\(^{2+}\)-permeable AMPA receptors in CA1 results in increasing its vulnerability to ischemic injury (Oguro et al., 1999). In addition, using focal (middle cerebral artery occlusion, MCAO) and global ischemia animal models, we found an increased association of p97 with GluR1 in these rats (data not shown). Thus alteration of p97 expression and its interaction with GluR1 may provide a new mechanism in regulating homomeric GluR1 receptor trafficking and function after ischemia insults. Therefore, future studies could include identifying signaling molecules that lead to increased p97-GluR1 association and hence, increase homomeric GluR1 AMPAR-mediated neuronal death.
4.4 References


APPENDICES

Appendix A: Publications

1. Published papers


2. Papers in preparation


NMDA-dependent long-term potentiation promotes differentiation and proliferation of neural stem cells. In preparation.

3. Abstracts


Appendix B: UBC research ethics board certificates of approval
## ANIMAL CARE CERTIFICATE

**Application Number:** A08-0247  
**Investigator or Course Director:** Yu Tian Wang  
**Department:** Medicine, Department of  
**Animals:**  
- Rats Sprague 0  
- Rats Sprague Dawley 100  

**Start Date:** April 1, 2008  
**Approval Date:** June 22, 2009  

### Funding Sources:

<table>
<thead>
<tr>
<th>Funding Agency</th>
<th>Funding Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canadian Institutes of Health Research (CIHR)</td>
<td>AMPA receptor regulation and synaptic plasticity</td>
</tr>
<tr>
<td>Michael Smith Foundation for Health Research</td>
<td>Postsynaptic regulation of neurotransmission</td>
</tr>
<tr>
<td>NeuroScience Canada Foundation</td>
<td>Synaptic repair by peptides interfering with protein-protein interactions critical for synaptic plasticity and maturation: a rational pharmacology for restoring cognitive and emotional function</td>
</tr>
<tr>
<td>Canadian Institutes of Health Research (CIHR)</td>
<td>LTD blocking peptide as a therapy for neuropsychiatric illnesses and stroke</td>
</tr>
<tr>
<td>Heart and Stroke Foundation of Canada</td>
<td>Investigation into molecular mechanisms mediating excitotoxicity - developing novel strategies in stroke treatment</td>
</tr>
<tr>
<td>Canadian Institutes of Health Research (CIHR)</td>
<td>Investigation into molecular mechanisms and physiological roles of activity-dependent AMPA receptor insertion</td>
</tr>
<tr>
<td>Howard Hughes Medical Institute</td>
<td>Investigation into the molecular mechanisms underlying synaptic plasticity</td>
</tr>
</tbody>
</table>
Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Role of hippocampal synaptic plasticity in cue-induced relapsing

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
**ANIMAL CARE CERTIFICATE**

**Application Number:** A06-0356

**Investigator or Course Director:** Yu Tian Wang

**Department:** Medicine, Department of

**Animals:**

| Rats Sprague Dawley 100 |

| Start Date: December 1, 2007 | Approval Date: February 17, 2009 |

**Funding Sources:**

<table>
<thead>
<tr>
<th>Funding Agency:</th>
<th>Canadian Institutes of Health Research (CIHR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Funding Title:</td>
<td>LTD blocking peptide as a therapy for neuropsychiatric illnesses and stroke</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Funding Agency:</th>
<th>Canadian Institutes of Health Research (CIHR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Funding Title:</td>
<td>LTD blocking peptide as a therapy for neuropsychiatric illnesses and stroke</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Funding Agency:</th>
<th>Canadian Institutes of Health Research (CIHR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Funding Title:</td>
<td>Role of hippocampal synaptic plasticity in cue-induced relapsing</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Funding Agency:</th>
<th>NeuroScience Canada Foundation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Funding Title:</td>
<td>Synaptic repair by peptides interfering with protein-protein interactions critical for synaptic plasticity and maturation: a rational pharmacology for restoring cognitive and emotional function</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Funding Agency:</th>
<th>NeuroScience Canada Foundation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Funding Title:</td>
<td>Synaptic repair by peptides interfering with protein-protein interactions critical for synaptic plasticity and maturation: a rational pharmacology for restoring cognitive and emotional function</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Funding Agency:</th>
<th>Canadian Institutes of Health Research (CIHR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Funding Title:</td>
<td>AMPA receptor regulation and synaptic plasticity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Funding Agency:</th>
<th>Canadian Institutes of Health Research (CIHR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Funding Title:</td>
<td>Role of hippocampal synaptic plasticity in cue-induced relapsing</td>
</tr>
<tr>
<td>Funding Agency</td>
<td>Heart and Stroke Foundation of British Columbia and Yukon</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>----------------------------------------------------------</td>
</tr>
<tr>
<td>Funding Title</td>
<td>Investigation into the molecular mechanisms mediating excitotoxicity - developing novel post-stroke therapies</td>
</tr>
<tr>
<td>Funding Agency</td>
<td>Canadian Institutes of Health Research (CIHR)</td>
</tr>
<tr>
<td>Funding Title</td>
<td>LTD blocking peptide as a therapy for neuropsychiatric illnesses</td>
</tr>
<tr>
<td>Funding Agency</td>
<td>Canadian Institutes of Health Research (CIHR)</td>
</tr>
<tr>
<td>Funding Title</td>
<td>Investigation into molecular mechanisms and physiological roles of activity-dependent AMPA receptor insertion</td>
</tr>
<tr>
<td>Funding Agency</td>
<td>Canadian Institutes of Health Research (CIHR)</td>
</tr>
<tr>
<td>Funding Title</td>
<td>Regulation of postsynaptic expression of GABA-A receptors</td>
</tr>
<tr>
<td>Unfunded title</td>
<td>N/A</td>
</tr>
</tbody>
</table>

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration  
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3  
Phone: 604-827-5111 Fax: 604-822-5093
# ANIMAL CARE CERTIFICATE

**Application Number:** A05-0827

**Investigator or Course Director:** Yu Tian Wang

**Department:** Medicine, Department of

**Animals:**

| Rats Fisher-344 64 |
| Rats Sprague-Dawley 240 |

**Start Date:** May 1, 2007  
**Approval Date:** June 4, 2009

**Funding Sources:**

<table>
<thead>
<tr>
<th>Funding Agency</th>
<th>Funding Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canadian Institutes of Health Research (CIHR)</td>
<td>Investigation into molecular mechanisms and physiological roles of activity-dependent AMPA receptor insertion</td>
</tr>
<tr>
<td>Canadian Institutes of Health Research (CIHR)</td>
<td>Role of hippocampal synaptic plasticity in cue-induced relapsing</td>
</tr>
<tr>
<td>Canadian Institutes of Health Research (CIHR)</td>
<td>Investigation into molecular mechanisms and physiological roles of activity-dependent AMPA receptor insertion</td>
</tr>
<tr>
<td>Canadian Institutes of Health Research (CIHR)</td>
<td>Role of hippocampal synaptic plasticity in cue-induced relapsing</td>
</tr>
<tr>
<td>Canadian Institutes of Health Research (CIHR)</td>
<td>Regulation of postsynaptic expression of GABA-A receptors</td>
</tr>
<tr>
<td>Canadian Institutes of Health Research (CIHR)</td>
<td>AMPA receptor regulation and synaptic plasticity</td>
</tr>
<tr>
<td>NeuroScience Canada Foundation</td>
<td>Synaptic repair by peptides interfering with protein-protein interactions critical for synaptic plasticity and maturation: a rational pharmacology for restoring cognitive and emotional function</td>
</tr>
</tbody>
</table>
The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
ANIMAL CARE CERTIFICATE

Application Number: A08-0807

Investigator or Course Director: Yu Tian Wang

Department: Medicine, Department of

Animals: Rats Sprague Dawley & Wistar 400

Start Date: May 21, 2009

Approval Date: June 15, 2009

Funding Sources:

Funding Agency: Canadian Stroke Network (CSN) - Networks of Centres of Excellence (NCE)
Funding Title: Targeting cell death cascades in the neuro vascular-inflammatory unit

Funding Agency: Various Sources
Funding Title: Molecular mechanisms of AMPA receptor cycling

Funding Agency: Howard Hughes Medical Institute
Funding Title: Investigation into the molecular mechanisms underlying synaptic plasticity

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: A new therapeutic that protects the brain against excitotoxic and ischemic neuronal injuries

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Investigation into molecular mechanisms and physiological roles of activity-dependent AMPA receptor insertion

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Role of hippocampal synaptic plasticity in cue-induced relapsing

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Regulation of postsynaptic expression and function of GABA-A receptors

Funding Agency: Heart and Stroke Foundation of British Columbia and Yukon
Funding Title: Investigation into the molecular mechanisms mediating excitotoxicity - developing novel
post-stroke therapies

**Funding Agency:** National Natural Science Foundation of China  
**Funding Title:** Role of hippocampal synaptic plasticity in cue-induced relapsing

**Funding Agency:** Canadian Stroke Network (CSN) - Networks of Centres of Excellence (NCE)  
**Funding Title:** Development of a stroke model in non-human primates

**Funding Agency:** UBC Faculty of Medicine  
**Funding Title:** New faculty start-up grant

**Funding Agency:** CHDI Foundation  
**Funding Title:** TREAT-HD: Translational Research on Excitotoxicity to Accelerate Therapies for Huntington's Disease

**Unfunded title:** N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

**A copy of this certificate must be displayed in your animal facility.**

Office of Research Services and Administration  
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3  
Phone: 604-827-5111 Fax: 604-822-5093