DEVELOPING NOVEL THERAPEUTICS FOR EXCITOTOXIC NEURONAL DEATH FOLLOWING STROKE

by

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ABSTRACT

Ischemic stroke is a major cause of death and disability in developed countries, and a major economic burden in the world. The mechanisms mediating stroke damage are likely multifactorial, with N-methyl-D-aspartate receptor (NMDAR) mediated excitotoxicity being an important factor. But NMDAR blockers are not clinically feasible due to their side effects and short therapeutic window. This doctoral dissertation discusses an ongoing effort to develop novel anti-excitotoxic therapeutics that can overcome these limitations. Our strategies are largely based on the premises that NR2A-subunit containing NMDARs (NR2ARs) are pro-survival whereas NR2B-subunit containing NMDARs (NR2BRs) are pro-death, and NR2BRs mediate neuronal death in large by activating a set of neuronal death signaling proteins.

Specific Findings: 1, we investigated whether stimulation of NR2ARs by the NMDAR co-agonist glycine rather than blocking NR2BRs would confer a wider therapeutic time window in an in vivo rat model of focal and global ischemia. Because we stimulated neuronal survival rather than blocked neuronal death, our therapeutic remained efficacious up to 6 h post-ictus - a time point when many known death signaling proteins downstream of NR2BRs were already activated. 2, we studied the death-signaling pathway of SREBP1 (sterol response element binding protein 1), a transcription factor downstream of NR2BR, in an in vivo rat model of focal ischemia. In the ischemic brain, we found SREBP1 activation and nuclear translocation due to the ubiquitination and degradation of its inhibitory partner Insig1 (protein encoded by insulin signaling gene1). Notably, the new therapeutic peptide Indip (Insig1 degradation inhibiting peptide) prevented neuronal death when administered 1 h pre- and 2 h post-ictus. Because we targeted a death-signaling protein rather than all signaling proteins downstream of NMDARs, our treatment would have fewer side effects than NMDAR blockers. 3, we developed a novel method to selectively knockdown death-signaling proteins downstream of NR2BRs by means of ubiquitin-tagged peptides. Because interference peptides in the past were limited to disrupting protein-protein interactions and post-translational modifications, this opened a new avenue to develop therapeutics for excitotoxicity following stroke.
PREFACE

PUBLISHED PAPERS PRESENTED IN DISSERTATION:


PUBLISHED ABSTRACTS PRESENTED IN DISSERTATION:


Most of the work presented in this dissertation is published, and as evident from the multiple authorships included in each publication, each paper is a joint collaboration between my colleagues and me. In the interest of space, however, only the work that I am directly involved with is presented in this thesis. Readers interested in other relevant work can refer to the publications listed above. A version of **CHAPTERS 1.2-1.4** was published as a review article (Lai et al. 2011b). I was responsible for the initial writing, which was then modified by my advisor Prof. Yu-Tian Wang. A modified version of **CHAPTER 1.5** was published as a News and Views article (Lai et al. 2010). Results in **CHAPTER 3** were published in Liu et al. (2007) and Lai et al. (2008). The animal work presented in Liu et al. (2007) was a joint effort between Yitao Liu, Dong Chuan Wu, and me, and the work presented in Lai et al. (2008) came from me and my summer/co-op undergraduate students Kaitlyn Hu, Monica Du, and David Ko. Results in **CHAPTER 4** were published in Lai et al. (2007 and 2009) and Taghibiglou et al. (2009). I was responsible for all animal work presented, and was chiefly responsible for the writings in Lai et al. (2007 and 2009) and partly responsible for the writing in Taghibiglou et al. (2009). Results in **CHAPTER 5** were published in Lai et al. (2011a). I was solely responsible for all the experimental work presented, and was jointly responsible for
the writing of the manuscript with my advisor. All animal works conducted were approved by the UBC ACC: including the MCAO protocol A07-0151 and the Global ischemia protocol A07-0155.
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<tr>
<td>ACC</td>
<td>Animal care committee</td>
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<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid</td>
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<td>AMPAR</td>
<td>AMPA receptor</td>
</tr>
<tr>
<td>APH</td>
<td>2-amino-7-phosphonoheptanoic acid</td>
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<tr>
<td>ARU</td>
<td>Animal research unit</td>
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<td>1,2-Bis(2-aminophenoxy)ethane-(N,N,N',N')-tetraacetic acid</td>
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<td>bovine serum albumin</td>
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<td>common carotid artery</td>
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<td>CREB</td>
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<td>(\gamma)-D-glutamylglycine</td>
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<td>D-MEM</td>
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<tr>
<td>DUB</td>
<td>De-ubiquitinating enzyme</td>
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<td>excitatory amino acid</td>
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<td>ethylene glycol-bis(2-aminoethyl)-(N,N,N',N')-tetraacetic acid</td>
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<td>mGFP</td>
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To my brother, parents, and grandparents

for their

unconditional love and support
CHAPTER 1: INTRODUCTION

1.1 Overview

Ischemic stroke is a major cause of death and disability in developed countries, and represents a major economic burden in the world (Danton and Dietrich, 2004; Gladstone et al., 2002; Lopez et al., 2006). Because neuronal death in the brain following stroke is an active and prolonged process (Dirnagl et al., 1999; Lipton, 1999), understanding the underlying death-signaling mechanism can lead to therapeutics that minimize stroke damage even when administered several hours to days after a stroke. To date, unfortunately, no such neuroprotective therapeutics is in clinical use.

The mechanisms mediating stroke damage likely involve multiple events (Besancon et al., 2008; Lee et al., 1999), and one of the events is the overactivation of glutamate receptors, particularly of the N-methyl-D-aspartate receptor (NMDAR) type, leading to “excitotoxicity” (Besancon et al., 2008; Lee et al., 1999; Rothman and Olney, 1995; Simon et al., 1984). In light of the critical importance of NMDARs, the 1980s and 1990s see the development of several potent NMDAR antagonists that are effective in reducing neuronal damage in experimental models of stroke (Boast et al., 1988; Choi, 1987a; Woodruff et al., 1987). Nevertheless, none of these compounds demonstrated definitive benefit compared to the placebo in large clinical trials (De Keyser et al., 1999; Devuyst and Bogousslavsky, 1999a, b; Wood and Hawkinson, 1997), in part because of their side effects and short therapeutic time window (post-ictus time to which the drug remains effective) (Gladstone et al., 2002; Wood and Hawkinson, 1997).
Extensive research in the last decade has significantly advanced our understanding of NMDAR functions under both physiological and pathological conditions, and mechanisms mediating these functions (Lai et al., 2011; Lai and Wang, 2010). In this introductory chapter, I will discuss the historical perspectives of and the recent advances in stroke research, with a specific focus on NMDARs, and why I think novel therapeutics developed during the course of my PhD training may shine where conventional treatments have failed.

1.2 Stroke and NMDA Receptors

One critical question in the stroke field has been why the brain is so susceptible to ischemic damage, especially in comparison to other tissues and organs in the body (Choi and Rothman, 1990). One explanation was that brain tissue contains high level of the neurotoxic excitatory neurotransmitter glutamate, and many neurons in the brain contain receptors that actively respond to these neurotransmitters. The link between the excitatory neurotransmitter glutamate and stroke damage was first proposed as early as 1959 by Van Harreveld when he discovered that topical application of glutamate (abundant in the brain extracts) induces spreading depression (SD) in the rabbit brain, and postulated that glutamate may “also” participate in similar SD-like cortical changes following stroke. Nevertheless, this “theory” initially went largely unnoticed, and many of the early studies on glutamate-mediated toxicity were driven by an interest in the toxicology of monosodium glutamate (MSG), a popular food additive and medicine at that time (Olney, 1969a, b; Olney and Ho, 1970; Olney and Sharpe, 1969). Finally in the 1980s, a series of pioneering studies solidified the role of glutamate in stroke damage, and further led to the development of many “anti-excitotoxic drugs” aimed to become effective stroke treatments.
1.2.1 Glutamate mediates excitotoxicity

Long before the role of glutamate as a neurotransmitter was clearly understood, Lucas and Newhouse (1957) made the pivotal observation that subcutaneous injection of MSG produces neuronal damage in the inner layer of the mouse retina. Two years later, Curtis et al. (1959) reported for the first time that glutamate is “excitatory”: that it can depolarize neurons, and thereby cause them to fire action potentials. Curtis et al. defined the depolarizing effect as “nonspecific”, because contrary to the principal excitatory neurotransmitter acetylcholine that only depolarize certain cell types, glutamate depolarized all neuronal types tested in their study. Subsequently, Olney and colleagues in a series of experiments confirmed that glutamate is indeed neurotoxic, and that the effect was not limited to the retina but also to the brain of mice, rats, rabbits, and monkeys, and using different routes of administration (including orally) (Olney, 1969a; Olney and Ho, 1970; Olney and Sharpe, 1969). Accordingly, he became a strong advocate against the regular use of MSG as a food supplement. Because glutamate is an “excitatory amino acid (EAA)” and other related EAAs are also neurotoxic, Olney coined the term “excitotoxins” to describe glutamate and other neurotoxic EAAs.

In studying glutamate's neurotoxic mechanism, Olney made several observations that eventually led to the “excitotoxicity hypothesis”: that glutamate mediates neuronal damage chiefly by inducing neuronal depolarization (excitation) (Rothman and Olney, 1986). First, glutamate analogues that were excitatory were also neurotoxic, and their toxicity corresponded to their potency at inducing neuronal excitation. Second, glutamate analogues that were not excitatory were also not neurotoxic, and antagonists of glutamate prevented both neuronal excitation and neurotoxicity. Third, rapid neuronal swelling, a pathological feature thought to underlie neurotoxicity, occurred only in the dendrites and the soma, whereabouts the
depolarization occurred. Although later studies led by Choi (1985, 1987b) and Garthwaite et al. (1986a, c; 1986a) demonstrated unequivocally that calcium, rather than sodium (and the depolarization/excitation), is required for glutamate-mediated neuronal death, the term “excitotoxicity” continued to be used to describe neurotoxicity caused by glutamate and other EAAs.

1.2.2 The calcium hypothesis

The calcium hypothesis, that glutamate’s neurotoxicity requires calcium, was first proposed by Berdichevsky et al. (1983) and Coyle (1983) based on their literature review that many cytotoxic processes in neuronal and non-neuronal tissues (ie. heart, muscle, and liver) require calcium (supporting the “calcium hypothesis”). Although two earlier studies have already made observation that glutamate induces calcium uptake, these studies have attributed the influx to be secondary to the activation of voltage-gated calcium channel (Heinemann and Pumain, 1980) and sodium-calcium exchangers (Cooke and Robinson, 1971). Berdichevsky et al. (1983) found that calcium readily accumulates in rat brain cortical slice when exposed to 5 min of neurotoxic EAAs such as NMDA and glutamate, but not when exposed to non-toxic analogues. More importantly, he reported that NMDA among other glutamate analogues is the most efficient EAA for inducing calcium accumulation, and suggested that the ionotropic glutamate receptors specifically activated by NMDA, now known as NMDARs, must underlie glutamate’s toxicity.

Nevertheless, the work by Berdichevsky et al. (1983) was solely correlative, and triggered much debate on whether glutamate induces neuronal death by “excitotoxic” mechanism or “calcium” mechanism. For instance, in a rebuttal to the study reported by Berdichevsky et al. (1983), Rothman (1985) and Olney & colleagues (1986; 1985)
independently showed that although calcium is indeed accumulated in neurons subjected to glutamate, preventing this accumulation by removing extracellular calcium does not attenuate glutamate-mediated rapid neuronal swelling (see above) that was thought to underlie glutamate’s toxicity. Instead, replacing sodium with a non-membrane permeable cation prevented glutamate-induced rapid swelling, and thus provided support for the “excitotoxicity hypothesis” (Olney et al., 1986; Price et al., 1985; Rothman, 1985). Moreover, in the absence of glutamate, inducing neuronal excitation by increasing potassium concentration in the media also induced rapid swelling. This later finding suggested that any source of excitation (depolarization) is potentially harmful.

The debate between the “excitotoxicity hypothesis” and the “calcium hypothesis” went on for several years. Rothman and Olney et al. independently demonstrated that chloride influx secondary to the initial depolarization triggered by glutamate (sodium) or potassium is required for the excitotoxic neuronal swelling (Olney et al., 1986; Price et al., 1985; Rothman, 1985). Yet around the same year, Choi (1985) reported that removal of calcium prevents a delayed form of glutamate-induced neuronal death, providing the first causative evidence that calcium may participate in glutamate’s neurotoxicity. Shortly thereafter, Garthwaite and colleagues (1986a, b, c; 1986a; 1986b) reproduced many of the earlier findings through a series of experimental studies, showing: (1) direct correlation between toxicity and excitation of several glutamate analogues (supporting the “excitotoxicity hypothesis”), (2) direct correlation between toxicity and calcium (supporting the “calcium hypothesis”), and (3) that extracellular calcium rather than sodium and chloride is chiefly required for delayed neuronal death (supporting the “calcium hypothesis”).

In reconciling the controversies, Choi (1987b) repeated his earlier experiment but this time paid particular attention to glutamate-mediated acute neuronal swelling, which was the
primary endpoint used by laboratories supporting the “excitotoxicity hypothesis”. Interestingly, he found that transient treatment of neurons with glutamate for 5 min induced two pathological features at different time points: (1) acute neuronal swelling that occurred immediately, and (2) delayed neuronal death that he found 24 h later. Importantly, he found that removal of sodium and chloride in the media only prevents acute neuronal swelling, but does not change the eventual neuronal death 24 h later. In contrast, removal of calcium actually exacerbated acute neuronal swelling, but prevented delayed neuronal death, and in which case the morphological (ie. swelling) and many of the physiological features of a normal undamaged neuron eventually recovered. This pioneering work laid the foundation for the “calcium hypothesis” of glutamate’s toxicity for many years to come. Around this same period, emerging experimental evidence from many groups begin to suggest that NMDARs, the most calcium permeable of the ionotropic glutamate receptors, are chiefly responsible for ischemic neuronal damage following stroke (see Chapter 1.2.4).

1.2.3 Modifications to the calcium hypothesis

The initial calcium hypothesis quickly evolved to become what may be called the “calcium overload hypothesis” (See Choi’s review (1988)): that glutamate mediates neuronal death by increasing intracellular calcium load, and that blocking multiple routes of calcium entry by a cocktail of calcium channel inhibitors may be more effective than blocking a single route of calcium entry. This hypothesis certainly did not hold true. As early as 1985, in an effort to rule out the “calcium hypothesis” in support of the “excitotoxicity hypothesis”, Olney and colleagues has already demonstrated that injection of nimodipine (blocker of voltage-gated calcium channel (VGCC)) exacerbates rather than attenuates NMDA or glutamate-induced neuronal death in mice in vivo (Price et al., 1985). A note-worthy modified version of the
“calcium overload hypothesis” was the “calcium set-point hypothesis”: that too much or too little calcium are both detrimental to neuronal health. The set-point hypothesis was initially proposed by Johnson and colleagues in 1989 when they showed that too little calcium is also harmful to cells (Koike et al., 1989), and in 1992 when they reviewed a myriad of experimental evidence supporting this point of view (Franklin and Johnson, 1992). Thereafter, Choi has also supported the new set-point hypothesis in his 1995 review (Choi, 1995).

In 1993, Tymianski et al. proposed the “distinct (calcium) pathway hypothesis”: that calcium source rather than (or in addition to) calcium load determines neurotoxicity. Specifically, they found that calcium loading induced by glutamate (through NMDARs) is especially neurotoxic, compared to equivalent calcium loading induced by high potassium (through VGCCs). From this result, they further proposed that some calcium-dependent death machineries must be physically bound to NMDARs. In 1994, they corroborated this hypothesis by showing that fast calcium chelator BAPTA is much more neuroprotective than the slow chelator EGTA (Tymianski et al., 1994). Indeed, between 1996 to 1999, several groups have reported evidence that NMDARs are specifically coupled to the neurotoxic enzyme nNOS through a scaffolding protein PSD95 (see Chapter 1.4.1 for detailed discussion), and that specific disruption of NMDARs from nNOS protects neurons against glutamate’s toxicity (Brenman et al., 1996; Christopherson et al., 1999; Kornau et al., 1995; Niethammer et al., 1996). Today, many other death-signaling proteins have been identified to directly bind to NMDARs (see Chapter 1.4). Thus, the “distinct pathway hypothesis”, more than the “overload hypothesis” or the “set-point hypothesis”, may be the most accurate derivative of the original “calcium hypothesis”.

1.2.4 Excitotoxicity following stroke

The first link between glutamate and ischemic damage came from Rothman’s (1983) surprising discovery that newly cultured hippocampal neurons lacking synapses are resistant to oxygen deprivation for up to 24 h, whereas matured cultured neurons with intact synaptic connections degenerate abruptly upon oxygen deprivation. Moreover, reducing synaptic activity by tetrodotoxin (TTX) or MgCl$_2$ prevented anoxic neuronal death in mature cortical neurons. He suggested that the synaptic release of the putative transmitter glutamate was required for anoxic damage. Indeed his later work in 1984 confirmed that blockade of ionotropic glutamate receptors with the drug γ-D-glutamylglycine (DGG) blocks both synaptic activity and anoxic neuronal damage in mature cultured neurons (Rothman, 1984). In the same year, Simon et al. (1984) demonstrated the first clear evidence that glutamate, in particular its action on NMDARs, is required for stroke damage. In this study, direct intracerebral injection of the NMDAR blocker 2-amino-7-phosphonoheptanoic acid (APH) into the rat brain prevented neuronal death following cerebral ischemia, suggesting that NMDAR blockers may be effective drugs for treating stroke. The following few years see the development of several NMDAR blockers for this purpose, as pharmaceutical companies aim for better selectivity, potency, efficacy, and membrane permeability (Boast et al., 1988; George et al., 1988; Germano et al., 1987; Kochhar et al., 1988; McDonald et al., 1987; Prince and Feeser, 1988; Woodruff et al., 1987).

1.3 Dual Roles of NMDARs in Death and Survival

Amid the findings that NMDARs are required for glutamate-mediated neuronal death in neurodegenerative diseases including seizure, stroke, Huntington’s, and other diseases, the late 1980s saw the development of many drugs that may act as potential antagonists at ionotropic
glutamate receptors. The enthusiasm was somewhat short-lived, as the rapid development of effective NMDAR antagonists quickly led scientists to discover many of their problems. In particular, Olney et al. (1989) while studying the neuroprotective effect of NMDAR blockers phencyclidine (PCP) and MK801 against kainic acid-induced seizure-related neuronal death found that seizure-related neuronal damage is indeed attenuated, but a new kind of neuropathology ensues. He then confirmed these findings in control non-seizured rats given these NMDAR blockers. These neuropathological features were later known as Olney’s lesions. Later work by him and others confirmed that NMDARs are indeed required for the well being of the brain and blockade can be detrimental to the developing (Ikonomidou et al., 1999; Pohl et al., 1999) and the injured brain (Ikonomidou et al., 2000).

In view of the “distinct pathway hypothesis”, that NMDARs are more neurotoxic than other calcium channels, scientists begin to question whether distinct NMDAR subpopulations can also determine neurotoxicity, and more specifically, whether this can explain the dual roles of NMDARs in neuronal death and survival.

1.3.1 The NMDAR location hypothesis

Given that some signaling or scaffolding proteins such as PSD95 (postsynaptic density protein-95) are distinctly located in the synapses (Cho et al., 1992), NMDARs at the synapse might be coupled to synapse-specific signaling proteins, thereby exerting functions distinct from extrasynaptic NMDARs. In support of this notion, Lu et al. (2001) provided the first proof-of-concept that distinct NMDAR subpopulations in the synapse and extrasynaptic sites can mediate opposing functional outcomes. They found that preferential activation of synaptic NMDARs mediates postsynaptic AMPAR (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid type ionotropic glutamate receptor) insertion, resulting in long-term
potentiation (LTP), a well characterized form of synaptic plasticity; by contrast, the activation of extrasynaptic NMDARs mediates AMPAR endocytosis, leading to the opposing synaptic phenomenon, long-term depression (LTD) (Lu et al., 2001) (for review on NMDAR and synaptic plasticity, see (Collingridge et al., 2010)). Several subsequent studies have provided support for this “NMDAR location” hypothesis in the field of neuronal survival and death (Hardingham et al., 2002; Leveille et al., 2008; Stanika et al., 2009; Xu et al., 2009; Zhang et al., 2007b). Specifically, synaptic NMDAR stimulation has prosurvival effects owing to its activation of the downstream cyclic-AMP response element-binding protein (CREB) signaling pathway; by contrast, the activation of extrasynaptic NMDARs mediates pro-death effects by attenuating the CREB pathway (Hardingham et al., 2002) (see also: Leveille, et al., 2008; Stanika, et al., 2009; Xu, et al., 2009; Zhang, et al., 2007 for further work by others, and review: Hardingham & Bading, 2010). Whether the “NMDAR location” hypothesis is instructive for explaining ischemic brain damage in in vivo animal models of stroke remains to be investigated. In this regard, it is interesting to note that memantine, a low-affinity uncompetitive NMDAR antagonist, can selectively block extrasynaptic NMDARs at specified doses, thereby protecting against neuronal death in a mouse model of Huntington’s disease (Milnerwood et al., 2010; Okamoto et al., 2009). Although memantine is also neuroprotective in stroke models, the concentrations used were much higher than the specified doses for a preferential blockade of extrasynaptic NMDARs (Aluclu et al., 2008).

The most important question concerning the “NMDAR location” hypothesis is how survival- and death-signaling proteins are compartmentalized to different subcellular locations. One possibility is that certain death-signaling proteins only bind to extrasynaptic NMDARs, but fail to bind to synaptic NMDARs due to steric hindrance by the extensive cluster of protein networks at the synapse. On the other hand, some survival-signaling proteins can be
preferentially compartmentalized to the synaptic sites through interactions with certain synaptic proteins in the postsynaptic density. Notably and contrary to this hypothesis, however, the best-characterized synapse-specific protein PSD95 (with only scarce presence at extrasynaptic sites (Cho et al., 1992; Petralia et al., 2010)) is required for NMDAR-mediated excitotoxic neuronal death (Aarts et al., 2002; Christopherson et al., 1999; Lai and Wang, 2010; Sattler et al., 1999; Zhou et al., 2010b) (see Chapter 1.4.1). Moreover, the neurotoxic role of PSD95 is consistent with earlier experimental evidence suggesting that synaptic NMDAR activity can be important for ischemic neuronal death during stroke (Rothman, 1984; Rothman, 1983; Tymianski et al., 1993). Although these controversies do not exclude the notion that synaptic NMDARs are primarily pro-survival, they do suggest that, at the very least, some synaptically localized NMDARs can exert pro-death effects, thereby having roles pathologically relevant to stroke damage.

1.3.2 The NMDAR subtype hypothesis

NMDAR subunits are classified into three subfamilies: NR1 (also called GluN1), NR2 (A-D; also called GluN2A-D) and NR3 (A and B; also called GluN3A,B) (Flint et al., 1997; Kirson and Yaari, 1996; Li et al., 1998; Stephenson, 2001; Stocca and Vicini, 1998). Native NMDARs are tetrameric complexes comprised two obligatory NR1 subunits with at least one type of NR2 subunit (Flint et al., 1997; Kirson and Yaari, 1996; Laube et al., 1998; Li et al., 1998; Schorge and Colquhoun, 2003; Stephenson, 2001; Stocca and Vicini, 1998; Ulbrich and Isacoff, 2007). Different NR2 subunits not only confer distinct electrophysiological and pharmacological properties on the receptors (Cull-Candy et al., 2001; Tovar and Westbrook, 1999), but also couple the receptor to different signaling machineries (Kohr et al., 2003; Ryan et al., 2008; Sheng and Pak, 2000; Traynelis et al., 2010) because of the structural diversity of
their carboxyl terminus (cytoplasmic tail) (Ryan et al., 2008). In addition, subunit compositions can influence the temporal and spatial distributions of the receptor (Cull-Candy et al., 2001; Tovar and Westbrook, 1999; Traynelis et al., 2010). The newborn forebrain is almost entirely populated by the NR2B-containing NMDARs (NR2BRs), but with developmental maturation, the synapses become increasingly populated by NR2A-containing NMDARs (NR2ARs), whereas extrasynaptic sites remain populated by NR2BRs (Tovar and Westbrook, 1999; Traynelis et al., 2010). Together, subtype-specific location and subtype-specific downstream signaling can confer subtype-specific NMDAR functional outputs.

1.3.2.1 Subtype hypothesis - Early work

Ever since the molecular cloning of different NMDAR subunits between 1991 and 1992, scientists have begun to investigate developmental changes in NMDAR expression and distribution, as well as their functional consequences pertaining to neuronal survival and death. Bessho et al. (1994) reported that early in development, cerebellar granule neurons contain low levels of NR2A, NR2B, NR2D, and very little NR2C. At this developmental stage, stimulation of NMDARs is largely pro-survival. In adult animals, these neurons contain much higher level of NR2A and NR2C, and stimulation of NMDARs is pro-death. They went on to demonstrate that NMDAR activity is required for the upregulation of NR2A, and that artificial induction of NR2A expression by stimulating NMDARs in young neurons sufficiently converts NMDARs from pro-survival receptors into pro-death receptors. From these results, they raised the possibility that the NR2A subunit is required for NMDAR-mediated neuronal death. In contrast, Mizuta et al. (1998) saw a developmental increase in NR2BR expression that corresponds to an increase in susceptibility to neuronal death, and suggested that NR2BRs rather than NMDARs containing other NR2 subunits (A, C-D) are
required for neuronal death. Mitani et al. (1998) reported that susceptibility of developing pontine nucleus to exogenous application of NMDA is greatest at 15 days postnatal, consistent with most prominent expression of NR2C-containing NMDARs (NR2CRs) at this time point. Therefore, they suggested that the functional switch between non-NR2C NMDARs and NR2CRs might be required for the developmental changes in susceptibility of pontine nucleus to NMDA stimulation. These early studies demonstrated how the expression of specific NMDAR subtype and the neuronal susceptibility to excitotoxicity could vary correspondingly depending on the tissue selected and the stage in the course of the development. However, because these studies are more or less correlative, causative relationships could not be formulated from these results.

1.3.2.2 Subtype hypothesis - Later work

The recent availability of subtype preferential antagonists (Auberson et al., 2002; Fischer et al., 1997; Liu et al., 2004a), mice lacking NR2A and NR2B NMDAR subunits, and proteomics techniques has allowed scientists to study the distinct functions mediated by the two major NMDAR subtypes in the adult forebrain where stroke most frequently occurs (Chen et al., 2008; DeRidder et al., 2006; Liu et al., 2007; Terasaki et al., 2010; Zhou and Baudry, 2006). In particular, several groups have reported that NR2ARs and NR2BRs are required for glutamate-mediated neuronal survival and death in both in vitro and in vivo models of stroke and traumatic brain injuries, respectively (Chen et al., 2008; DeRidder et al., 2006; Liu et al., 2007; Terasaki et al., 2010; Zhou and Baudry, 2006). These results based on pharmacological antagonism are further corroborated by results obtained from genetic mice lacking either NR2A or NR2B subunits (Liu et al., 2007). Detailed pharmacological analyses further reveal that the selective stimulation of synaptically localized NR2BRs, however scarce (Cull-Candy...
et al., 2001; Tovar and Westbrook, 1999; Traynelis et al., 2010), triggers excitotoxic neuronal
death; moreover, the activation of extrasynaptic NR2ARs, despite their subcellular locations,
promotes neuronal survival (Liu et al., 2007). These results support the notion that it is the
NMDAR’s subtype rather than its subcellular location that dictates whether the receptor is
pro-survival or pro-death. In addition, several survival-signaling pathways mediated by
CREB (Chen et al., 2008; Terasaki et al., 2010), PI3K (phosphoinositide 3-kinase) (Lee et al.,
2002) and Kidins220/ARMS (kinase-D-interacting substrate of 220 kDa) (Lopez-Menendez et
al., 2009), have been linked to NR2AR activation, whereas most death-signaling pathways are
selectively activated by NR2BRs (Aarts et al., 2002; Ning et al., 2004; Sattler et al., 1999;
Taghibiglou et al., 2009; Tu et al., 2010). Because different NR2 subunits can bind distinct
signaling proteins via direct protein-protein interactions (Kohr et al., 2003; Sheng and Pak,
2000), the “NMDAR subunit” hypothesis might provide a better explanation for how survival-
and death-signaling proteins are compartmentalized to distinct NMDAR subpopulations.
Indeed, many NMDAR death-signaling proteins identified thus far form complexes with
NR2BRs through either direct or indirect protein-protein interactions (Aarts et al., 2002; Ning
et al., 2004; Sattler et al., 1999; Tu et al., 2010).

Several remaining questions warrant future research and further modifications to the
“NMDAR subtype” hypothesis. First, most of the evidence supporting the “NMDAR
subtype” hypothesis came from experiments using subtype-specific antagonists (Chen et al.,
2008; DeRidder et al., 2006; Liu et al., 2007; Terasaki et al., 2010; Zhou and Baudry, 2006).
However, the selectively of some of these antagonists has been questioned (Berberich et al.,
2005; Neyton and Paoletti, 2006; Weitlauf et al., 2005). This may be addressed by the future
development of more specific NR2AR agonists or antagonists. Second, in addition to the
classic heterodimeric NR2ARs and NR2BRs, cortical neurons might also express
heterotrimeric NMDARs containing both NR2A and NR2B subunits (Traynelis et al., 2010). How these receptors fit into the “NMDAR subunit” hypothesis remains to be determined, but early evidence suggests that unlike pure NR2ARs, this special NMDAR subpopulation can be pro-death (O'Donnell et al., 2006). Third, under certain experimental conditions, NA2ARs can contribute to neuronal death and NR2BRs can promote neuronal survival (Martel et al., 2009; von Engelhardt et al., 2007). Thus, the roles of specific NMDAR subtypes will undoubtedly vary depending on the nature of the signaling complexes that are bound to the receptor at that particular stage of development, the area of the brain, and the model of disease.

1.3.3 A unified hypothesis

The exact ways by which NMDARs differentially confer different and sometimes opposing cellular functions may be more complicated than we can justify comprehensively and unequivocally with currently available experimental data. Nevertheless and while some controversies remain, the “NMDAR location” and the “NMDAR subtype” hypotheses are actually highly complementary. Given that in the adult forebrain where stroke most frequently occurs, NR2ARs and NR2BRs are preferentially localized at the synaptic and extrasynaptic sites, respectively (Cull-Candy et al., 2001; Liu et al., 2007; Tovar and Westbrook, 1999), stimulating synaptic and extrasynaptic NMDARs would predominantly activate NR2AR-dependent neuronal survival and NR2BR-mediated neuronal death pathways, respectively and vice versa. From these points of view, a unified hypothesis can be synthesized (illustrated in the FIGURE 1-1). Normal synaptic transmission activates predominantly NR2ARs, resulting in the maintenance of neuronal survival via the activation of the NSC (neuronal survival signaling complex) immediately downstream of these receptors. During stroke, glutamate
surges to the extrasynaptic sites primarily due to the reverse operation of the glutamate transporters (Colleoni et al., 2008; Rao et al., 2001; Rossi et al., 2000), where stimulation of NR2BRs mediates neuronal death by the activation of the NDC (neuronal death signaling complex) associated with these receptors (Lai et al., 2011). Note that here, we loosely define the NSC and NDC to include all neuronal survival- and death-signaling proteins that closely associate with the NMDAR channel pore either through spatial compartmentalization to the synapses or extrasynaptic sites, or through direct or indirect protein-to-protein interactions with NMDAR itself. Their close association with the NMDAR allows the efficient translation of calcium influx through the receptor channel into their respective functional outputs with high levels of spatial and subunit specificity.

1.4 Death Signaling Proteins in the NDC

The specific functions of any particular NMDAR subtype depend on the type of signaling proteins bound to the C-terminus of its NR2 subunit. This is supported by the finding that genetic-deletion of specific NR2 carboxyl-terminus results in subtype-specific loss of important NMDAR functions such as prenatal survival (NR2B tail-deletion), synaptic plasticity (NR2A tail-deletion), contextual memory (NR2A tail-deletion), and motor coordination (NR2C tail-deletion) (Sprengel et al., 1998). Thus, one way to identify death-signaling proteins downstream of NR2BRs is to identify which proteins are directly bound to the NR2B subunit, and then by means of pharmacological and genetic methods test whether the identified NR2B-bound protein is indeed excitotoxic. In fact, some death-signaling proteins that directly bind to the NR2BR have already been identified, and they include: (1) PSD95, (2) DAPK1 (death associated protein kinase-1), and (3) PTEN (phosphatase and tensin homolog deleted on chromosome 10).
Disrupting the binding of NR2B with these death-signaling proteins by an interference peptide that mimics the amino-acid-sequence of NR2B carboxyl-tail (Tat-NR2B-CT, where the “Tat” sequence makes the peptide cell membrane and blood-brain-barrier (BBB) permeable) prevents excitotoxic neuronal death following ischemia in vitro and in vivo, with the added benefit of not affecting NR2BR channel activity and other signaling proteins. Moreover, because each of these death-signaling proteins binds to a different segment of the NR2B-CT, several different versions of Tat-NR2B-CT peptides have been developed to specifically disrupt the binding of NR2BRs to each of these death-signaling proteins (Aarts et al., 2002; Lai and Wang, 2010; Tu et al., 2010; Zhou et al., 2010b).

1.4.1 The NR2B-PSD95-nNOS signaling complex

The first set of death-signaling proteins to be identified is probably the NR2B-PSD95-nNOS signaling complex (FIGURE 1-2) (Aarts et al., 2002; Christopherson et al., 1999; Lai and Wang, 2010; Sattler et al., 1999; Zhou et al., 2010b). PSD95 is a synaptic scaffolding protein that binds NMDARs and brings several signaling molecules to the vicinity of the NMDAR channel pore (Cho et al., 1992; Christopherson et al., 1999; Jurado et al., 2010; Petralia et al., 2010). It has three PDZ-domains for binding to proteins containing specific PDZ-ligands such as the NR2B-CT, and the N terminus beta-finger of neuronal nitric oxide synthase (nNOS) (FIGURE 1-2A) (Brenman et al., 1996; Christopherson et al., 1999; Lai and Wang, 2010). The simultaneous binding of PSD95 to NR2B-CT and nNOS is required for NMDAR-mediated production of the neurotoxic molecule nitric oxide (Christopherson et al., 1999; Garthwaite et al., 1988; Kiedrowski et al., 1992). Inhibiting nNOS activity by gene-deletion or by pharmacological means prevents NMDAR-dependent excitotoxicity in cultured neurons (Dawson et al., 1996; Samdani et al., 1997; Sattler et al., 1999; Sun et al., 2009), and
attenuates cerebral infarct and behavioral deficit in stroke animals (Samdani et al., 1997; Zhou et al., 2010b). Moreover, direct application of nitric oxide donors in cultured neurons is sufficient to induce neurotoxicity, suggesting that nitric oxide produced by nNOS can be a death signal downstream of NR2BRs (Sattler et al., 1999; Zhou et al., 2010b).

Importantly, this NMDAR NDC formation is enhanced by the stimulation of the NR2BRs during stroke, thereby forming a vicious cycle wherein NR2BR-mediated nitric oxide production can be progressively amplified (Zhou et al., 2010b). Because both NMDAR and nNOS perform many important physiological functions in the brain, therapeutics that specifically disrupt this complex formation are expected to have fewer neurological side effects than do conventional drugs that directly block NMDAR or inhibit nNOS. The first compound of this kind is the 20 amino acid-long Tat-NR2B9c peptide (FIGURE 1-2B) (Aarts et al., 2002), whose primary sequence includes the membrane-transducing domain of the HIV1 Tat protein that renders the peptide membrane permeable (Vives et al., 1997), and the nine amino acid residues encoding the PSD95 binding domain of the NR2B-CT. This peptide disrupts NR2B–PSD95 interaction by competing with native NR2B-CT for binding to PSD95; therefore, it has no effect on NMDAR channel activity or NR2A–PSD95 interaction (Aarts et al., 2002). To increase bioavailability and facilitate drug administration, two recent studies developed non-peptidic small molecules that mimic the interference peptides in disrupting this complex (FIGURE 1-2C) (Florio et al., 2009; Zhou et al., 2010b). The first study (Florio et al., 2009) reported a small molecule IC87201, modified from a lead compound identified through high-throughput screening of a chemical library of 150,000 small molecules. The second study (Zhou et al., 2010b) took a rationalized drug design approach, and designed and synthesized a de novo small molecule ZL006 based on the molecular properties of the interaction between nNOS and PSD95.
As expected, these new therapeutics (Tat-NR2B9c and ZL006) can effectively reduce stroke damage and lack the neurological side effects commonly associated with NMDAR antagonists or nNOS inhibitors (Lai and Wang, 2010; Zhou et al., 2010b). Notably, one of these compounds, Tat-NR2B9c, is already being tested for neuroprotective efficacy and safety in a Phase 2 clinical study (ISRCTN# NCT00728182). The upcoming results form this study, if positive, would warrant future development of more NMDAR-based therapeutic peptides targeting downstream death signaling proteins in the NDC.

1.4.2 The NR2BR-PTEN signaling complex

The tumor suppressor PTEN (Baker, 2007) is a well characterized cell death-promoting molecule that was recently identified as a crucial component of the NDC (Gary and Mattson, 2002; Lee et al., 2009; Liu et al., 2010; Ning et al., 2004; Zhang et al., 2007a; Zhang, 2009). PTEN is recruited to the NR2BRs (but not to the NR2ARs) via interactions with the NR1 subunit (Ning et al., 2004) and with PSD95 in a manner dependent on NMDAR calcium influx (Jurado et al., 2010). Moreover, the NR2BR-PTEN association potentiates NR2BR channel activity (Ning et al., 2004), and this can further enhance their association. This self-propagating mechanism, along with the well-known inhibition of the PI3K survival-signaling pathway by PTEN contributes to NR2BR-mediated neuronal death following stroke (Ning et al., 2004).

Interestingly, ischemia in animals subjected to stroke also triggered a time-dependent translocation of PTEN into the nucleus (Zhang, 2009). Consistent with selective binding of PTEN to NR2BRs, this nuclear translocation of PTEN requires NR2BR, but not NR2AR, stimulation. Unlike in other cell types where monoubiquitination of PTEN at residues K13 and K289 is thought to be required for nuclear translocation and tumor suppressing effects
(Baker, 2007), only the K13 site is required for PTEN nuclear translocation and consequent excitotoxic neuronal injuries in cortical neurons (Zhang, 2009). In an effort to translate this exciting finding to clinical use, an interference peptide Tat-K13 was developed. It contains the amino acid residues flanking the K13 ubiquitination site on PTEN and the membrane transduction domain of the HIV1 Tat protein, which renders the peptide membrane permeable (Brooks et al., 2005). When given to rats subjected to focal ischemia, Tat-K13, but not the control Tat-K289, strongly protects the rat brain against cerebral infarction even when administered 6 h after the stroke onset (Zhang, 2009). These studies add to the growing evidence supporting that the specific inhibition of the NDC can confer much wider therapeutic time windows than can blocking NMDARs with conventional blockers.

1.4.3 The NR2B-DAPK1 signaling complex

A recent study identified DAPK1 as a new component of the NDC (Tu et al., 2010). DAPK1 is a member of a serine/threonine kinase family well known for its role in cell death (Bialik and Kimchi, 2006). In a search for the most prevalent molecules in the NR2B-associated NDC following stroke, the total mix of signaling machineries directly or indirectly bound to NR2BRs before and after a stroke insult to the rat brain were analyzed by means of co-immunoprecipitation with anti-NR2B antibodies followed by mass spectrometry (Tu et al., 2010). The results demonstrated that following stroke, DAPK1 is activated and recruited to the NR2BR through its direct binding to the amino acid residues 1292-1304 of the NR2B carboxyl terminus (NR2B-CT_{1292-1304}). This direct DAPK1–NR2B interaction enables DAPK1-mediated phosphorylation of the NR2B subunit at serine-1303, thereby potentiating NR2BR activity. To selectively and competitively disrupt the DAPK1–NR2B interaction, an interference peptide, Tat-NR2B-CT_{1292-1304}, was developed whose primary sequence includes the
membrane traducing domain of the HIV1 Tat protein and the DAPK1-binding domain of NR2B (Tu et al., 2010). This peptide not only prevents DAPK1-mediated NR2B subunit phosphorylation and thereby NR2BR activity potentiation, but also attenuates excitotoxic neuronal injury. Importantly, when applied systemically to animals, Tat-NR2B-CT prevents ischemic brain damage following stroke (Tu et al., 2010).

It is especially important to note that DAPK1 is not associated with NR2BRs under basal conditions, but is only recruited to the NDC of NR2BRs following stroke insults. This stroke-induced recruitment of NDC molecules can, in part, explain why NMDAR signaling is normally benign (FIGURE 1-3A), but under pathological conditions such as stroke, NMDAR stimulation contributes to neuronal death (FIGURE 1-3B). Additional research is also required to further characterize the detailed mechanism by which DAPK1 mediates neuronal death (FIGURE 1-3B). Because DAPK1 potentiates NR2BR activity, it would inevitably also promote neuronal death by potentiating functions of “calcium-activated death-signaling proteins” in the NDC (FIGURE 1-3B, “calcium current”); however, this is probably not the only mechanism. Given the fundamental role of DAPK1 as a pro-apoptotic protein and tumor suppressor (Bialik and Kimchi, 2006), the recruitment of DAPK1 to the NDC following stroke is probably self-sufficient for inducing neuronal death by a direct activity on “phosphorylation-dependent death-signaling proteins” in the NDC via its kinase activity (FIGURE 1-3B, “? arrow”). An example of such a death-signaling protein is protein kinase D (PKD), whose phosphorylative activation by DAPK1 is in turn required for its phosphorylation-mediated activation of the death-signaling protein c-Jun N-terminal kinase (JNK) (Eisenberg-Lerner and Kimchi, 2007).
1.5 Beyond the NDC

The molecular cascade leading to excitotoxicity is a multi-step process. It begins with stimulation of the extrasynaptic/NR2B NMDARs by glutamate, resulting in the activation of death signaling proteins in the NDC immediately downstream of NMDARs (Lai et al., 2011). These death signaling proteins in the NDC then go on to activate other death signaling proteins further downstream and beyond the NMDAR/NDC. For instance, activation of the death-signaling protein p38 (see Chapter 1.4.3) during stroke is thought to be secondary to the stimulation of NR2B-PSD95-nNOS in the NDC. Moreover, as mentioned earlier, activation of JNK (see Chapter 1.4.3) is in part secondary to the activation of DAPK1 in the NDC. Therefore, the new targets for NMDAR-mediated excitotoxic neuronal death are not only limited to the NDC, but also include the death-signaling proteins further downstream of the NDC. Because neurons can die of slow processes over hours to days following a stroke insult (Dirnagl et al., 1999; Lipton, 1999), these newly identified death-signaling proteins and pathways further downstream of the NMDAR are promising targets for novel NMDAR-based therapeutics with wider therapeutic time windows.

1.5.1 The calpains family of death-signaling proteases

This family of calcium-activated cysteine proteases plays a major role in translating the calcium influx of NMDAR into neuronal injuries (Brorson et al., 1995; DeRidder et al., 2006; Koumura et al., 2008; Lopez-Menendez et al., 2009; Taghibiglou et al., 2009; Zhou and Baudry, 2006). In line with the “NMDAR subtype” and “NMDAR location” hypotheses, calpains are only activated by NR2BRs, but not NR2ARs (DeRidder et al., 2006; Lopez-Menendez et al., 2009; Zhou and Baudry, 2006), and only by extrasynaptic, but not synaptic, NMDARs (Xu et al., 2009). The inhibition of calpains (Brorson et al., 1995; Koumura et al.,
2008; Zhou and Baudry, 2006) or their downstream death-signaling pathways (Taghibiglou et al., 2009; Xu et al., 2009) is strongly protective against NMDAR-mediated neuronal damage in vitro (Brorson et al., 1995; Taghibiglou et al., 2009; Xu et al., 2009; Zhou and Baudry, 2006) and in vivo (Koumura et al., 2008; Taghibiglou et al., 2009). In support of the notion that blocking these downstream death-signaling proteins can confer a wider therapeutic time window than can blocking NMDARs, the calpain inhibitor SNJ-1945 protects mice against permanent focal ischemia even when the treatment is delayed for up to 6 h post-stroke (Koumura et al., 2008).

These cysteine proteases exert their neurotoxic actions mainly by the proteolytic destruction/inhibition of survival-signaling proteins (Lopez-Menendez et al., 2009; Xu et al., 2009; Xu et al., 2007) and by the proteolytic activation of death-signaling proteins (Taghibiglou et al., 2009; Xu et al., 2007). First, calpains directly cleave survival-signaling proteins STEP-61 (striatal-enriched tyrosine phosphatase-61) (Xu et al., 2009) and Kidins220/ARMS (Lopez-Menendez et al., 2009), thereby contributing to extrasynaptic NMDAR- (Xu et al., 2009) and NR2BR- (Lopez-Menendez et al., 2009) mediated neuronal death, respectively. Therefore, the inhibition of STEP61 cleavage by the Tat-STEP peptide rescues the STEP61-mediated inhibition of p38 death signaling and protects neurons against NMDAR-mediated excitotoxic damage (Xu et al., 2009). Second, the calpain-mediated cleavage of p35 into p25 and the subsequent activation of cyclin-dependent kinase 5 contribute to glutamate-mediated excitotoxic neuronal death following stroke (Lee et al., 2000). Likewise, the NR2BR-mediated proteolytic activation of the pro-death protein SREBP1 (sterol response element binding protein-1) requires calpain activity (Taghibiglou et al., 2009); however, it remains unclear whether calpains directly cleave SREBP1 (see Chapter 1.4.4). Third, calpains cleave mGluR1α (metabotropic glutamate receptor 1α), thereby
converting this natively pro-survival glutamate receptor into a pro-death receptor (Xu et al., 2007). Indeed, the Tat-mGluR1α peptide protects neurons against excitotoxic death in *vitro* and *in vivo* (Xu et al., 2007).

### 1.5.2 AMPAR trafficking as an excitotoxic process

One of the best characterized functional outputs of NR2BR is regulated AMPAR endocytosis, a common mechanism responsible for the expression of various forms of LTD (Kim et al., 2005; Li et al., 2010; Tigaret et al., 2006); nevertheless, it was not until recently that this was recognized as an essential step downstream of NMDAR-mediated excitotoxic neuronal death (Wang et al., 2004). The inhibition of AMPAR endocytosis by two structurally different inhibitors of clathrin-mediated endocytosis prevents NMDAR-mediated neuronal death without affecting NMDAR activity (Wang et al., 2004). More importantly, the specific inhibition of AMPAR endocytosis with an interference peptide GluR23Y, whose sequence is derived from the GluR2 tyrosine phosphorylation sites required for regulated AMPAR endocytosis and LTD (Ahmadian et al., 2004), prevents neurons against excitotoxic damage *in vitro* (Wang et al., 2004). This finding is in agreement with earlier studies showing that clathrin-mediated endocytosis is required for retinal neuronal degeneration (Dolph, 2002); moreover, it raised the intriguing possibility that LTD can contribute to neuronal death. Whether this process is involved and whether the GluR23Y peptide is effective in animal models of stroke *in vivo* remain to be investigated.

How AMPAR endocytosis mediates excitotoxic neuronal death remains unclear. First, whereas an earlier study reported that AMPAR endocytosis mediates neuronal death both by enhancing the caspase-3 death-signaling pathway and inhibiting the PI3K survival-signaling pathway (Wang et al., 2004), a recent study showed that increased caspase-3 activity and
decreased PI3K signaling is actually required for AMPAR endocytosis (Li et al., 2010). Together, these two studies suggest that an amplification of death signaling via positive feedback interplay between AMPAR endocytosis and caspase-3 following NR2BR activation is required for excitotoxic neuronal death. Second, clathrin-mediated rhodopsin endocytosis promotes its interaction with the clathrin adaptor protein arrestin, and this rhodopsin-arrestin interaction induces retinal neuronal death in Drosophila (Dolph, 2002). Given that β-arrestin also interacts with activated JNK3 (McDonald et al., 2000) (see Chapter 1.4.3), it is possible that a clathrin adaptor protein such as β-arrestin also activates and/or recruits death-signaling molecules such as JNK3 to endocytosed AMPARs, thereby mediating AMPAR endocytosis-dependent neuronal apoptosis. Third, because GluR2-containing AMPARs are specifically endocytosed following NR2BR stimulation, the endocytosis could lead to a compensatory increase in the expression of surface GluR2-lacking AMPARs following stroke (Liu et al., 2004b; Noh et al., 2005). These GluR2-lacking receptors are characterized by high calcium permeability, and as such, they might contribute to neuronal death following ischemic stroke (Liu et al., 2004b; Noh et al., 2005).

1.5.3 MAPKs in neuronal survival and death

Mitogen-activated protein kinases (MAPKs) are traditionally known for transducing extracellular signals from neurotransmitters and hormones to the nucleus, resulting in gene expression changes (Pearson et al., 2001). There are three main types of MAPKs: (i) the extracellular signal-regulated kinases (ERKs) that are generally pro-survival and are activated by growth factors and other survival factors, (ii) the p38 protein kinase (also known as CSBP/RK/MPK2) and (iii) JNK (also called stress-activated protein kinase (SAPK)), which mediate cell death in response to inflammatory cytokines and cellular stress. Consistent with
their roles in neuronal survival and death, synaptic/NR2AR stimulation induces ERK activation (Kim et al., 2005), whereas extrasynaptic/NR2BR stimulation inhibits ERK activation (indirectly via synGAP (Kim et al., 2005) or DAPK1 (Eisenberg-Lerner and Kimchi, 2007)) and induces p38 activation (Xu et al., 2009). Moreover, upregulation of the NR2B subunit in the spinal cord has been implicated in JNK activation (Guo et al., 2009).

1.5.3.1 Early death-signaling by p38 kinase

p38 is activated via phosphorylation in cultured neurons by excitotoxic NMDAR stimulation, and in agreement with the pro-death role of p38, inhibition of p38 with selective inhibitors SB203580 and SB239063 prevents NMDAR-mediated neuronal death in in vitro (Cao et al., 2005; Kawasaki et al., 1997; Legos et al., 2002) and in vivo (Barone et al., 2001) models of stroke and excitotoxicity. Notably, recent studies have demonstrated that p38 is probably a downstream effector of the NDC given that NMDAR-mediated p38 activation requires intact NR2B-PSD95-nNOS formation (Cao et al., 2005; Soriano et al., 2008) and the calpain-mediated proteolysis of STEP-61, yielding STEP-33 (Xu et al., 2009). Nevertheless, its therapeutic potential is hindered by its early activation time course (Cao et al., 2004; Cao et al., 2005). NMDAR-mediated p38 activation peaks around 5 min post-treatment in cultured neurons, and becomes greatly reduced by 30 min and completely abolished by 60 min. Likewise, SB203580 only protects cultured neurons against excitotoxicity when applied 30 min before excitotoxic glutamate-treatment, but not when applied 30 min post-glutamate treatment (Cao et al., 2005). In marked contrast, nNOS inhibitors (Brorson et al., 1995), PSD95 inhibitor Tat-NR2B9c (Aarts et al., 2002), and calpain inhibitor MDL-28170 (Brorson et al., 1995) remain strongly protective against NMDAR-mediated excitotoxicity even when applied up to 60 min post-glutamate treatment, a time-point when p38 is no longer active (Cao
et al., 2004; Cao et al., 2005). This suggests that p38 is probably not the only death signaling protein downstream of NR2B-PSD95-nNOS and NR2B-calpain, and other delayed/prolonged death signaling proteins may explain the wide therapeutic window of nNOS inhibitors, Tat-NR2B9c, and calpain inhibitors.

1.5.3.2 Delayed death-signaling by JNK

JNK may be one of “slowest” NMDAR death signaling pathways found so far. It is activated as early as 1 h following ischemic stroke in rats, and remains strongly activated for as long as 24 hrs post-stroke (Borsello et al., 2003). JNK is inactive when bound to JNK-binding domain (JBD20, residues 143-163) of the JNK-interacting protein-1 (JIP-1) at the 4 critical amino acid residues: R156, P157, L160, L162 (Barr et al., 2002; Bonny et al., 2001) and that this inactivation can be artificially mimicked by the JBD20 fragment (Barr et al., 2002; Bonny et al., 2001; Borsello et al., 2003). Based on these, an interference peptide Tat-JBD20 is developed as a specific JNK inhibitor. Impressively, Tat-JBD20 is highly selective: inhibiting JNK at 2.5-25μM while having no effect on ERK2, p38, PKC, P34, CamK, and PKA at 500μM (Borsello et al., 2003). Selective inhibition of JNK with D-JNKI-1 (a D-amino acid variant of Tat-JBD20) protects cultured cortical neurons against NMDA-induced neuronal death in vitro and reduces ischemic brain damages in rat models of both transient and permanent focal ischemia (Borsello et al., 2003; Esneault et al., 2008; Hirt et al., 2004). Notably, and consistent with the long-lasting activity of JNK following stroke, inhibition by D-JNKI-1 has a relatively long post-stroke therapeutic time window of up to 12 h following transient focal ischemia (Borsello et al., 2003; Esneault et al., 2008), and 3 h following permanent focal ischemia (Hirt et al., 2004).
1.5.4 SREBP1 as a pro-death transcription factor

Although SREBP1 usually controls lipid biosynthesis genes (Goldstein et al., 2006), it was recently identified as a downstream death-signaling protein for NR2BR-mediated excitotoxic neuronal death following stroke (Taghibiglou et al., 2009). Because transcriptional activities can contribute to “slow” neuronal death signaling, pro-death transcription factors are promising targets for developing novel stroke therapeutics that reduce neuronal death with a prolonged therapeutic window. A recent non-biased screen (Taghibiglou et al., 2009) identified SREBP1 as a transcription factor whose activation by NR2BRs is required for neuronal death following stroke. The stimulation of NR2BRs, but not NR2ARs, triggers calcium-dependent ubiquitination and proteasomal degradation of INSIG1 (protein encoded by insulin induced gene1), an important inhibitory binding partner that normally retains inactive SREBP1 in the endoplasmic reticulum (ER) (Goldstein et al., 2006), thereby allowing SREBP1 to travel to the Golgi apparatus where it is cleaved to generate the active N terminus of SREBP1 (nt-SREBP1). nt-SREBP1 then translocates into the nucleus where it carries out transcriptional activities required for delayed neuronal death (Taghibiglou et al., 2009). As expected, the suppression of SREBP1 signaling by either oversupplying cholesterol or siRNA-mediated knockdown protects cultured neurons against excitotoxic/ischemic neuronal death in vitro (Taghibiglou et al., 2009).

Detailed mechanisms underlying SREBP1-mediated neuronal damage remain to be established. SREBPs are the major transcription factors that regulate the expression of a large number of gene products involved in cellular cholesterol and lipid biogenesis (Goldstein et al., 2006), and metabolic alterations in some of these lipid products were recently implicated in mediating neuronal damage following stroke insults (Adibhatla et al., 2006; Siesjo and Katsura, 1992). In addition, SREBP1 also regulates the expression of proteins not directly
involved in lipid metabolism, including G proteins (Park et al., 2002) and voltage-gated ion channels (Park et al., 2008). Thus, SREBP1 can contribute to neuronal damage via a mechanism independent of, or in addition to, alterations in lipid metabolism.

1.6 Non-Neuronal Neurotoxic Mechanisms

The preceding sub-chapters describe how NMDARs may mediate neuronal death via calcium-influx-dependent activation of intra-neuronal death-signaling proteins. Nevertheless, the non-neuronal mechanisms of NMDAR-mediated neuronal death should not be overlooked. While it is conventionally thought that NMDARs are only expressed in neurons, research in the past decade has demonstrated clear evidence that glial cells also express functional NMDARs and that these receptors can be important for their physiology and pathology (Karadottir et al., 2005; Krebs et al., 2003). Notably, ischemia in the brain induces functional expression of NR2BRs in astrocytes (Krebs et al., 2003) and activates constitutively expressed NMDARs in oligodendrocytes (Karadottir et al., 2005). Given the ischemia-specific expression of NMDARs in astrocytes and ischemia-mediated stimulation of NMDARs in oligodendrocytes, the role of these “neuron supporting cells” in stroke damage and recovery are expected to be substantial. Not only can stimulation of NMDARs on glial cells directly contribute glial damage, it also triggers the death of surrounding neurons normally supported by these cells. Moreover, glial cells can contribute to inflammatory injury following stroke in an NMDAR-dependent manner. Direct intracerebral application of NMDAR agonist triggers activation of astrocytes and microglia in the brain, where these cells in turn induce inflammatory responses to cause neuronal death (Ryu et al., 2007). In addition to glial cells in the brain, leukocytes from the blood stream may enter the brain when the BBB becomes leaky following stroke (Bolton and Perry, 1998; Ryu et al., 2007). Indeed, direct infusion of NMDAR agonists into
the adult rat striatum induces the recruitment of active leukocytes from the blood stream into the brain (Bolton and Perry, 1998; Ryu et al., 2007). Importantly, neutralization of these inflammatory cells with anti-serum prevents NMDAR-mediated inflammation and excitotoxic neuronal death (Ryu et al., 2007).

1.7 Therapeutic Strategies for Stroke Intervention

Ischemic stroke is a leading cause of death and disability, for which there is no effective treatment (Danton and Dietrich, 2004; Gladstone et al., 2002; Lopez et al., 2006). Therefore, research and development of stroke therapeutics are urgent matters. When a cerebral artery becomes occluded during an episode of ischemic stroke, the deficiency in blood supply to meet the high energy demand of the brain triggers a cascade of events that eventually lead to progressive neuronal death and neurological deficits (Elijovich and Chong, 2010). Some of these events, particularly NMDAR-mediated excitotoxic events, are reviewed in the preceding chapters (Chapters 1.2-1.5) and in other review articles (Lai et al., 2011; Lai and Wang, 2010; Lee et al., 1999). As indicated in the title of this dissertation, the primary objective of my study is to develop novel therapeutics for excitotoxic neuronal death following stroke. To provide a comprehensive rationale to my approach, the stroke treatment that is currently available for clinical use and those that are undergoing clinical trials, as well as some of their major limitations are further discussed in this chapter.

From ischemia to excitotoxicity, neuronal death following stroke can be minimized by either relieving the vascular occlusion using vascular-based therapeutics (Chapter 1.6.1), or by inhibiting the cascades of events leading to neuronal death using neuroprotection-based therapeutics (Chapters 1.6.2 and 1.6.3).
1.7.1 tPA and its limitations

The ischemic brain may recover some of its blood supply by producing endogenous tissue plasminogen activator (tPA) that dissolves the clot responsible for the vascular occlusion (Elijovich and Chong, 2010). Indeed, the only treatment presently used in the stroke clinic is the recombinant tPA, which complements endogenous tPA to more thoroughly dissolve the occlusive clot (Elijovich and Chong, 2010). If given within 3 h post-ictus, exogenous tPA significantly improves stroke outcome. However, this therapeutic comes with high risks (Rosell et al., 2008): (1) in some ischemic stroke patients, tPA transforms the disease into a more severe and often fatal hemorrhagic stroke, and (2) in patients that already had hemorrhagic stroke, who may present clinical symptoms indistinguishable from ischemic stroke patients, tPA is almost always fatal. Therefore, within tPA’s short therapeutic time window (3 h post-ictus), patients need to be admitted to a hospital, diagnosed with stroke, scanned by x-ray computed tomography to rule out any signs of hemorrhage, and then finally administered intravenously with tPA. In marked contrast, neuroprotection-based therapeutics can be given to patients regardless of whether they have ischemic or hemorrhagic stroke, and should be effective in both cases. Moreover, because neuronal death following stroke can take up to days and weeks to fully manifest (Dirnagl et al., 1999; Lipton, 1999), these therapeutics can be designed to have very wide therapeutic time window - remaining effective even when administered many hours to days after stroke.

1.7.2 NMDAR blockers and their limitations

Neuronal death following stroke is a multi-factorial process (Besancon et al., 2008; Lee et al., 1999), and as discussed extensively in the preceding chapters, NMDAR-mediated excitotoxicity induced by glutamate is one of the primary factors (Choi, 1987a; Gotti et al.,
In light of this, several potent NMDAR blockers were developed with early preclinical success in preventing neuronal damage in experimental stroke models *in vitro* and *in vivo* (Boast et al., 1988; Choi, 1987a; Woodruff et al., 1987). Nevertheless, none of these drugs demonstrated definitive benefit compared to the placebo when tested in large clinical trials (Gladstone et al., 2002; Lee et al., 1999; Wood and Hawkinson, 1997).

### 1.7.2.1 Clinical failure of NMDAR blockers

Many explanations for the failure of NMDAR antagonists in clinical studies have been proposed by clinicians and scientists. These include poor experimental design, insufficient sample size because of heterogeneous patient populations, a lack of unified standards for outcome measures, side effects that limit the administration of effective doses and delay in patient admission and diagnosis, which prohibits early treatment (see reviews (Corbett and Nurse, 1998; Gladstone et al., 2002; Lee et al., 1999; Wood and Hawkinson, 1997)). Two of these factors are emphasized in this dissertation as they may be overcome by the novel therapeutics proposed here. First, NMDAR blockers are only effective when given before or soon after stroke onset (Gladstone et al., 2002; Wood and Hawkinson, 1997); it is thought that once NMDAR’s *downstream death signals* are activated, blocking NMDARs at the receptor level is no longer effective. In the clinic, however, patients are usually admitted many hours post-stroke. Secondly, NMDARs are essential receptors in the brain responsible for many neurological processes. Blocking NMDARs at the receptor level inhibits all the *downstream functional outputs*, resulting in neurological side effects like psychomimetic symptoms and memory impairment (Palmer, 2001; Wood and Hawkinson, 1997).
1.7.2.2 NR2BR-specific blockers and limitations

As discussed extensively in Chapter 1.3, NMDARs are also important for neuronal survival and development, and neuronal recovery after injury. This may explain why in some clinical trials, NMDAR blockers exacerbate rather than improve stroke outcome (Albers et al., 2001; Lees et al., 2001). Because NR2ARs are pro-survival and NR2BRs are pro-death, selective NR2BR antagonists may retain the neuroprotective effect yet confer fewer side effects compared to nonselective NMDAR blockers. In fact, one selective NR2BR blocker CP-101,606 (troxoprodil) has thus far demonstrated safety and tolerability, in addition to therapeutic efficacy, in small double-blinded placebo-controlled Phase II studies (Bullock et al., 1999; Merchant et al., 1999), while its Phase III trial remains ongoing. That being said, even the most selective NR2BR blockers may not be ideal for clinical use. Aside from pathological function during stroke, these pro-death receptors also carry important physiological functions, such as in the extinction of fear memory, manifestation of stress response, and learning of new memory. Thus, important adverse reactions may have been overlooked at lower doses in smaller clinical trials, but are expected to manifest with greater (and more effective) dose and treating larger patient population - even with the most selective NR2BR blocker. More importantly, like the nonspecific NMDAR antagonists, the therapeutic efficacy of NR2BR antagonists are limited by their narrow time window of opportunity. In an animal model of focal ischemic stroke, for instance, the highly selective NR2BR antagonist Ro 25-6981 is only neuroprotective when given prior to, but not 4.5 h after the onset of ischemia (see Chapter 3 for results). This limitation on the time window makes any NMDAR blockers, whether or not selective for NR2BRs, not ideal for clinical use.
1.7.3 Interference of NMDAR signaling by synthetic peptides

In an effort to circumvent the problems and limitations discussed above, many recent research studies have aimed to target death-signaling proteins downstream of NR2BRs, rather than the receptor *per se*. Since there are many downstream cellular signaling proteins, it is possible to tease out which ones are responsible for NR2BR-mediated excitotoxicity, and which ones are responsible for important physiological functions. By specifically targeting the downstream death signaling proteins, and avoiding functional signaling proteins, it may be possible to develop therapeutics that are equally effective compared to NR2BR blockers but with much fewer side effects. Moreover, like conventional NMDAR blockers, NR2BR blockers have narrow time window of opportunity due to the sequential nature of biochemical reaction cascades. That is, once the downstream death signals are activated, it is too late to block NR2BR-signaling at the receptor level. Therefore, blocking these downstream death signals may offer much wider, or at least more delayed, time window of opportunity compared to receptor blockers.

Many intracellular signaling cascades do not occur by molecule-receptor-binding as with inter-cellular communication, and as such, most intracellular biochemical cascades are without a selective drug inhibitor. In these cases, *de novo* biologics (protein/peptide-based therapeutic agents) that interfere with intracellular signaling can be developed. Because intracellular signaling often involves (1) protein-protein interactions and (2) post-translational modifications, it is possible to disrupt intracellular signaling cascades by introducing a small interference peptide that resembles the sequence of a target protein. This biologic would either (1) disrupt protein-protein interaction in a competitive manner, or (2) act as a pseudo-substrate for the supposed post-translational modification. Once the interference peptide sequence has been identified, these therapeutic peptides can be rendered cell membrane and
BBB permeable by fusion with the membrane transduction domain (MTD; ie. YGRKKRRQRRR-peptide) derived from the HIV 1 Tat protein (Brooks et al., 2005; Vives et al., 1997), or simply with the 11-amino acid residues-long poly-arginine sequence (ie. RRRRRRRRRRRR-peptide) (Hsieh et al., 2011).

Many recent studies have adapted this therapeutic strategy. They have identified specific death signals downstream of NR2BRs, and accordingly designed effective Tat-fusion peptides to disrupt downstream death signals from NR2BRs without affecting normal receptor function. As explained earlier, two important objectives of finding a downstream target are to (1) increase the time window of therapeutic efficacy, and to (2) reduce side effects associated with nonspecific inhibition of other signaling pathway. Many of these recently discovered biologics (ie. Tat-fusion peptides) are proven effective in animal models of stroke, with reduced side effects and/or greater therapeutic time window. One particular note-worthy biologic is Tat-NR2B9c (also called NA-1) that disrupts NR2B and PSD95 interaction (see Chapter 1.4.1). To the best of our knowledge, it is the first reported use of Tat-fusion peptide to directly target death signals downstream of NMDARs in stroke intervention, and the first Tat-fusion biologic to reach human clinical study for stroke (Visit www.strokecenter.org for up-to-date information on human stroke trials). As of the writing date of this manuscript, the Phase II clinical study of Tat-NR2B9c remains ongoing (ISRCTN# NCT00728182).

1.8 Rationale and Hypotheses

NMDAR functions largely depend on the activation of signaling complexes that are brought adjacent to the channel pore either through direct protein–protein interactions between one or more components of the signaling complex and the NMDAR (Aarts et al., 2002; Christopherson et al., 1999; Ning et al., 2004; Sattler et al., 1999; Tu et al., 2010) or by other
subcellular compartmentalization mechanisms (Hardingham and Bading, 2010; Hardingham et al., 2002; Leveille et al., 2008; Lu et al., 2001; Stanika et al., 2009; Xu et al., 2009; Zhang et al., 2007b). Normal activity of NMDARs primarily activates the NSC and other functional signaling complexes involved in mediating neuronal functions including neuronal circuit maturation, learning and memory and other behavioral functions (FIGURE 1-4). However, excessive NMDAR stimulation can also activate the NDC, especially when the NDC molecules are recruited to the NMDAR under pathological conditions such as stroke (Tu et al., 2010; Zhou et al., 2010b). The stimulation of the NDC under pathological conditions results in neuronal death (FIGURE 1-4A). Current treatments based on NMDAR antagonism block all signaling pathways downstream of NMDARs. The blockade of NMDAR neurological function can result in intolerable side effects such as psychomimetic effects and memory loss (Wood and Hawkinson, 1997; Zhou et al., 2010b). Moreover, the inhibition of the NSC under certain conditions can explain why the use of NMDAR blockers has exacerbated stroke outcome in some clinical trials (Albers et al., 2001; Lees et al., 2001) (FIGURE 1-4B). These undesirable actions have limited the clinical use of NMDAR blockers at the concentrations required to reduce ischemic damage.

Newly developed therapeutics should thus act by either enhancing NSC signaling (Hardingham et al., 2002; Liu et al., 2007; Terasaki et al., 2010) and/or selectively blocking NDC signaling (Aarts et al., 2002; Christopherson et al., 1999; Ning et al., 2004; Sattler et al., 1999; Tu et al., 2010) without affecting other NMDAR signaling pathways, thereby resulting in therapeutic efficacy without the many side effects (FIGURE 1-4C). Moreover, because conventional NMDAR antagonists target the earliest step of NMDAR death signaling, they become ineffective shortly following stroke when the NDC death-signaling cascade has already been activated. In marked contrast to this, many of the new therapeutics targeting the
NDC or signaling steps further downstream continue to be efficacious even when administered to experimental animals many hours after stroke (Aarts et al., 2002; Liu et al., 2007; Tu et al., 2010; Zhang, 2009; Zhou et al., 2010b).

1.8.1 Objective 1: Synaptic/NR2A NMDAR stimulation

Preclinical and clinical stroke studies more or less focus on investigating the different pathological events leading ischemia to neuronal death (Dirnagl et al., 1999; Lipton, 1999). Energy deprivation due to the ischemia results in a depolarizing ionic imbalance, which triggers massive glutamate release due to reverse function of the glutamate transporters (Kanthan et al., 1995; Rossi et al., 2000). The overstimulation of AMPARs and NMDARs by glutamate, together with the initial ionic imbalance, contributes to fast and immediate necrosis (Choi, 1996). Calcium-influx from various sources, including NMDARs, contributes to slow and delayed apoptosis (Choi, 1996). Other contributors to ischemic neuronal death include oxygen and nitrogen free radicals, mitochondrial dysfunction, and inflammatory damage (Besancon et al., 2008; Lee et al., 1999). Notably, drugs targeting these different processes are limited by their therapeutic time window. It is too late to block glutamate release after it has been released, and it is too late to block NMDARs when their downstream signaling pathways are already activated. This lack of time window is a major contributing factor for the massive failure of multiple experimental compounds in over a hundred clinical stroke studies (Gladstone et al., 2002).

As discussed in Chapter 1.3, synaptic/NR2A NMDARs and extrasynaptic/NR2B NMDARs have differential roles in promoting neuronal survival and death, respectively (Hardingham et al., 2002; Liu et al., 2007). This leads to our working hypothesis that promoting neuronal survival by stimulating synaptic/NR2A NMDARs can be an effective
strategy for post-stroke treatment, compared to conventional NMDAR blockers. Here, because we are stimulating neuronal survival signaling rather than blocking neuronal death pathways, our treatment should remain beneficial as long as neuronal death persists. Moreover, because NR2ARs protects against NMDAR-dependent as well as non-NMDAR-dependent neuronal death (Liu et al., 2007), our treatment will not only counter NMDAR-mediated excitotoxicity following stroke, but also other contributors to ischemic neuronal death such as free radicals, mitochondrial dysfunction, and inflammatory damages.

1.8.2 Objective 2: Inhibiting SREBP1 death signaling

Excitotoxicity during ischemia begins with the release of glutamate to the extrasynaptic sites, resulting in the activation of NR2BRs and the death-signaling proteins downstream of these receptors. Although several potent NMDAR antagonists are developed, they are only effective at preventing neuronal death when given to experimental animals before or soon after a stroke. It is thought that once the downstream death-signaling proteins become activated, these drugs become no longer effective. Therefore, it is better to directly target a critical downstream death-signaling protein rather than the receptors per se. Theoretically, the time window for drug administration is proportional to how far downstream a therapeutic target is; as such, drugs that target death signals that are further downstream have wider or more delayed time window (Lai et al., 2011). Since slow and delayed neuronal function often involves transcriptional regulation, we seek to identify the key transcription factors responsible for NMDAR-mediated excitotoxicity (Taghibiglou et al., 2009). According to our DNA-protein binding assay, SREBP1 is one such death-promoting transcription factor (Taghibiglou et al., 2009).

Because activation of SREBP1 by NR2BR is delayed for up to 6 h post-excitotoxic
stimulation, this death-signaling protein is indeed very far downstream of the receptor (Taghibiglou et al., 2009). Thus, we hypothesize that preventing neuronal death by inhibiting SREBP1 activation can offer wider time window for drug administration, compared to conventional NMDAR blockers or NR2BR antagonists. Since NR2BR-mediated SREBP1 activation requires insig1 ubiquitination and degradation, we are able to selectively interfere with NR2BR-to-SREBP1 signaling with a pseudo-substrate peptide with sequence resembling insig1 ubiquitination sites (Gong et al., 2006): insig 152-161 (GEPHKFKREW), and as detailed in Chapter 4, this peptide indeed prevents NR2BR-mediated insig1 degradation, SREBP1 activation, and stroke damage.

1.8.3 Objective 3: Novel method for protein knockdown

The preceding chapters discuss many of the death signaling proteins downstream of NR2BRs that are responsible for neuronal death following stroke, and many Tat-linked peptidic biologics that are developed to interfere with (1) protein-protein interaction and (2) post-translational protein-modification required for neuronal death. However, there exists no method to selectively knockdown targeted death-signaling proteins via protein-degradation pathways. Such methodology would be especially useful for targeting protein whose signaling mechanism remains unknown, or that the signaling mechanism does not involve protein-protein interaction or post-translational modifications. Given that protein-protein interaction sites of many proteins are already well characterized, and in situ hybridization libraries exist for quick commercial identification of novel protein-protein interaction sites of any given recombinant protein, it is nowadays simple to design synthetic biologics, proteins or peptides, that will specifically bind to any targeted protein of interest. This raises the possibility that addition of a ‘protein-degradation signal’ to these biologics may allow targeted degradation of
their specified partner protein.

The main mechanism of protein degradation in the mammalian cells is via the ubiquitin-proteasome system (UPS) (Finley and Chau, 1991). In this system, proteins destined for degradation are tagged with a signal protein called ubiquitin in a process called “ubiquitination”. This triggers the delivery of the protein for degradation by the proteasome (about 80% of proteins) or the lysosome (about 20% of proteins). In the present study, we hypothesize that *ubiquitin-tagged proteins, in addition to their own degradation, can facilitate the degradation of non-tagged proteins via protein-protein interaction*. This finding will facilitate the development of a novel method to selectively knockdown proteins via targeted degradation. Given the wide spread utilization of DNA knockout and mRNA knockdown protocols, this method would be of great interest not only to stroke researchers but all biology researchers in general.
NMDARs are calcium-permeable ionotopic glutamate receptors that mediate many different neuronal functions in the brain, including opposing functions like neuronal survival and death. In the adult brain where stroke most frequently occurs, the two major subtypes of NMDARs are (1) those containing NR2A subunits (orange) primarily in the synapses, and (2) those containing NR2B subunits (green) primarily in extrasynaptic sites. Whereas the synaptic NMDAR/2A subpopulation activates downstream NSC (NMDAR survival signaling complex) leading to neuronal survival, and extrasynaptic NMDAR/2B subpopulation activates downstream NDC (NMDAR death signaling complex) resulting in neuronal death under pathological conditions such as stroke.

(Source: Lai et al., 2011. Trends in Molecular Medicine)
FIGURE 1-2 The deadly association of the NR2B–PSD95–nNOS signaling complex.

Dissociation of the NR2B-PSD95-nNOS signaling complex prevents NMDAR-mediated production of the neurotoxic molecule NO, reducing stroke damage.  

A. The NR2B subunit (orange) of NMDAR forms a multimeric protein complex with PSD95 and nNOS.  

B. Tat-NR2B9c dissociates PSD95-nNOS from NR2BRs by disrupting NR2B-PSD95 interaction.  

C. ZL006 dissociates nNOS from NR2B-PSD95 by disrupting PSD95-nNOS interaction.

(Source: Lai et al., 2011. Trends in Molecular Medicine)
FIGURE 1-3 Recruitment of DAPK1 to the NR2B subunit following stroke.

A. The NR2B-associated NDC (NMDAR death signaling complex) is not active under normal conditions.  
B. DAPK1 is recruited to NDC through its direct binding to the carboxyl-terminus of NR2B during stroke, resulting in NDC activation either by potentiating NR2B activity or stimulating other NDC components.  
C. Tat-NR2BCT prevents the recruitment of DAPK1 to NDC and subsequent activation of NDC by disrupting NR2B-DAPK1 interaction.

(Source: Lai et al., 2011. Trends in Molecular Medicine)
A. NMDARs are calcium-permeable ionotropic glutamate receptors. They mediate many important functions in large due to the many signaling complexes directly bound to these receptors. For instance, they bind to NMDAR neuronal survival complex (NSC, blue) to help maintain neuronal survival in the brain. But they also bind to NMDAR neuronal death complex (NDC, red) which contribute to brain damage in the event of a stroke. B. Conventional NMDAR blockers inhibit both NDC and NSC pathways, and this may explain their failure in clinical studies. Moreover, because they block the very first step of NMDAR signaling at the surface receptor level, they become ineffective when the downstream NDC is already activated. C. New therapeutics described here will either stimulate the NSC or inhibit the NDC downstream of these receptors.

(Source: Lai et al., 2011. Trends in Molecular Medicine)
CHAPTER 2: MATERIALS AND METHODS

2.1 Animal Model of Focal Ischemic Stroke

2.1.1 Experimental animals

Male Sprague-Dawley rats (300 - 350 g, Charles River) were used in this study. The animals were housed in groups (2 rats per cage) in a laboratory animal room at the Animal Research Unit (ARU) of the University of British Columbia (UBC) Hospital for at least one week prior to experimental use, and they were housed individually following surgical preparation. They had free access to rat pellet chow and water. All experiments conducted were approved by the Animal Care Committee (ACC) of UBC (see MCAO protocol A07-0151).

2.1.2 Middle cerebral arterial occlusion (MCAO)

Anesthesia was induced with 4% isoflurane in a nitrous oxide/oxygen (70:30%) mixture in a rat anesthesia chamber, and thereafter maintained with 2.0 - 2.5 % isoflurane in a nitrous oxide/oxygen (70:30%) mixture. Body temperature was monitored with a rectal probe throughout the course of each surgery, and was recorded immediately after induction of anesthesia and again following the surgery. The rectal probe was connected to an automated heating bed, which was set to maintain body temperature at around 37°C. Blood oxygen and heart rate were also monitored by a pulse oximeter, with paw sensor attached to the left hindlimb.

Each rat was subjected to reversible MCAO by a suture-insertion method modified from that originally developed by Longa et al. (1989). The digastric, the omohyoid, and the
sternomastoid muscles were exposed through two consecutive midline incisions to the skin and the underlying connective tissue, respectively, while taking care not to wound the glands near the area of operation. The digastric, the omohyoid, and the sternomastoid muscles were isolated and retracted by means of 3-0 silk sutures to facilitate operation on the right common carotid artery (CCA), the external carotid artery (ECA), and the internal carotid artery (ICA). The CCA was carefully isolated from the adjacent vagal nerve and the omohyoid muscle. A loose 5-0 silk suture was placed around the CCA, and pulling of the CCA in the proximal direction facilitated operation on the ECA and ICA. The superior thyroid artery and the occipital artery branching off the ECA were isolated and coagulated. Thereafter, the distal end of the ECA was tied off with a 5-0 silk suture. The pterygopalatine artery branching off the ICA was found by further dissection in the distal direction of the ICA, and it was tied off with a 5-0 silk suture. Care was taken not to damage the glossopharyngeal nerve lying above the origin of the pterygopalatine artery.

After pulling the CCA via the loose suture to prevent blood flow and clipping the ICA with an aneurysm clip to prevent blood backflow, a small incision (or puncture) was made on the ECA between the tied suture and the carotid bifurcation. A “MCAO occluder”, which was a 30 mm length of 3-0 nylon suture with its tip rounded by a heated wooden stick, was introduced into the lumen of the ECA with its tip lying in the carotid bifurcation. Note that, to be sure that the occluders used in the studies were equally and consistently round and smooth, each occluder was checked under a dissection microscope prior to experimental use. The ECA was then tied with a 5-0 silk suture close to the carotid bifurcation to prevent bleeding, and the pulling of the CCA and the aneurysm clip on the ICA were released. The ECA was then cut and reflected to facilitate introduction of the occluder down the ICA. After the occluder had gone ~ 21 mm from the bifurcation down the ICA, a small resistance was felt.
This was indicative that the round tip of the occluder had reached and occluded the origin of the right middle cerebral artery. The surgical wound was closed by means of suturing, and the rat was allowed to recover from anesthesia during the occlusion period.

To induce reperfusion, the rat was anesthetized as described above. Rectal temperature was again measured via a rectal probe, and recorded before and after the surgery. The suturing of the skin was removed. The occluder was gently pulled back and removed, and the ECA was tied more tightly to prevent bleeding. The wound was again closed by means of suturing, and the rat was allowed to recover.

2.1.3 Neurological score

Each rat was subjected to six behavioral tests described by Belayev L et al. (1996), which incorporated the postural reflex test developed by Bederson et al. (1986) and the forelimb placing tests developed by De Ryck et al. (1989). These tests included (1) the postural reflex test, (2) the visual forward, (3) the visual sideways, (4) the tactile forward, (5) the tactile sideways, and (6) the proprioceptive placing tests. Each of the six tests were assigned a score of 0 for normal behaviour, 1 for partial deficit, and 2 for complete deficit, and the six test scores were summed to give a total neurological score of 12 with a minimum of 0 for normal rat behaviour.

During the postural reflex test (also called the “hang test”), the rat was suspended by holding its tail (Bederson et al., 1986). A normal rat (score = 0) would spread its arms, rather than curled up to one side, and a rat with partial deficit (score = 1) would curl up repetitively only to its left side. A complete deficit (score = 2) was characterized by the rat holding its grip while curling up. To perform the forelimb placing tests, the rat was suspended by grabbing it from its lower back (De Ryck et al., 1989). A visual stimulus was achieved by moving the rat
closer to the edge of a table in the forward and sideways direction. A tactile stimulus was achieved by gently touching the front paw of the rat with the edge of a table without the rat seeing it. A proprioceptive stimulus was applied by slowing placing the suspended rat onto the centre of a table with its front paws landing on the table first. In all the above five placing tests, a normal rat (score = 0) would exhibit complete placing of its front paws immediately after the stimulus. A rat with partial deficit (score = 1) would exhibit incomplete and/or delayed placing of the left paw (due to the MCAO on the right side of the brain), and a rat with complete deficit (score = 2) would exhibit no placing of the left paw.

2.1.4 Histology and infarct measurements

To quantify brain infarction volume, each rat was euthanized by an overdose of urethane (2 g/kg, i.p.). The head was bluntly displaced with a pair of scissors, and the skull and the overlaying skin were carefully peeled off with a pair of bone cutter designed for this purpose. The brain was gently removed, and coronally sectioned into six 2-mm-thick slices (from rostral to caudal, first to sixth). Each section was stained by bathing in a 2% 2,3,5-triphenyltetrazolium (TTC) solution in phosphate-buffered saline (PBS) for 10 min, and then fixed in a 4% paraformaldehyde solution in PBS. The PBS used contained either (mM, at pH 7.4): NaCl (119), KCl (4.7), KH2PO4 (1.18), MgSO4 (1.17), NaHCO3 (24.9), EDTA (0.023), CaCl2 (1.6), dextrose (11.1), or (mM, at pH 7.4): NaCl (137), KCl (2.7), Na2HPO4 (4.3), KH2PO4 (1.47). Thereafter, pictures of the brain slices were taken with a digital camera, and volumes of the infarcted tissue and the whole brain were quantified by multiplying the area (measured with the free computer software – Image J developed by the National Institutes of Health (NIH)) by the thickness (2 mm in all cases) of each slice.
2.1.5 Criteria for exclusion

To reduce variability in stroke damage due to surgical inconsistency between animals, a preset guideline was made to exclude possible outliers. There were two major criteria for excluding animals from experimental studies, and all exclusions were made while the investigator was blinded to the treatment groups. First, since neurological deficits during the occlusion period represented neuronal inactivity rather than neuronal death, it would not be affected by neuroprotective drugs. Thus, rats with poor neurological deficits during the occlusion period were considered poorly occluded, and were excluded. Second, intracerebral hemorrhage due to mechanical injury by the insertion of the ‘MCAO occluder’ could greatly exacerbate and complicate stroke outcome. Animals that displayed signs of hemorrhage in the brain identified following tissue isolation were excluded from the study.

2.2 Animal Model of Global Ischemia

2.2.1 Experimental animals

Male Sprague-Dawley rats (300 - 400 g, Charles River) were used in this study. The animals were housed in groups (2 rats per cage) in a laboratory animal room at the ARU of the UBC Hospital for at least one week prior to experimental use, and they were housed individually following surgical preparation. They had free access to rat pellet chow and water. All experiments conducted were approved by the ACC of UBC (see Global ischemia protocol A07-0155).

2.2.2 Global ischemia

Anesthesia was induced by a bolus injection of pentobarbital (65 mg/kg, i.p.), and thereafter maintained by supplemental doses of pentobarbital (~30% original dose) as required. Body
temperature was monitored with a rectal probe throughout the course of each surgery, and was recorded immediately after induction of anesthesia and again following the surgery. The rectal probe was connected to an automated heating bed, which was set to maintain body temperature at around 37°C. Blood oxygen and heart rate were also monitored by a pulse oximeter, with paw sensor attached to the left hindlimb.

Each rat was subjected to 10-min global ischemia by bilateral carotid arterial occlusion coupled to hypotension (decreasing blood pressure to 30-35 mmHg), modified from that reported by Smith et al. (1984). First, a midline neck incision was made, and both CCAs were carefully isolated from the adjacent vagal nerve and the omohyoid muscle. A 5-0 silk suture was placed around each CCA to facilitate its occlusion later on. Second, both femoral arteries were isolated from their adjacent femoral veins and femoral nerves, and cannulated with polyethylene-50 (PE-50) tubings filled with heparinized saline (25U heparin per mL of saline). The tubing attached to the left femoral artery was connected to a pressure transducer, whose signal was amplified and recorded onto a laptop computer. From this point on, blood pressure was monitored and recorded until the end of the operation. The tubing attached to the right femoral artery was then connected to a 10-mL syringe filled with 1-mL heparinized saline. Third, to induce global ischemia, 0.5 mL of the 1-mL heparinized saline was injected into the right femoral artery, followed by withdrawing of the blood until arterial pressure dropped to between 30-35 mmHg. This typically required the withdrawing of 7 to 10 mL of blood. The CCAs were then occluded by means of aneurysm clips (note that, this was facilitated by the 5-0 sutures previously placed around the CCAs), and this marked the beginning of the 10-min of global ischemia.

Following the 10-min transient global ischemia period, the aneurysm clips were quickly removed. Removal of the aneurysm clips marked the end of the 10-min period. The arterial
blood was then re-infused slowly back via the right femoral artery over a period of 1 to 2 min. The animals were placed under a heated blanket during the recovery from the surgery.

2.2.3 Electrophysiology in vivo

Anesthesia was induced by a bolus injection of pentobarbital (65 mg/kg, i.p.), and thereafter maintained by supplemental doses of pentobarbital (~30% original dose) as required. To allow induction of global ischemia during the recording sessions, each rat received the preparatory surgery for global ischemia (see Chapter 2.2.2) prior to being fixed onto a stereotaxic frame (head: around -2.0 mm) for electrophysiological recording. Body temperature was monitored with a rectal probe throughout the course of each surgery, and was recorded immediately after induction of anesthesia and again following the surgery. The rectal probe was connected to an automated heating bed, which was set to maintain body temperature at around 37°C. Blood oxygen and heart rate were also monitored by a pulse oximeter, with paw sensor attached to the left hindlimb.

The skull was exposed by a single midline incision to the overlaying skin, and uncovered by means of bulldog clips to the connective tissues and skin. The bregma and lambda were identified, and three holes were drilled through the skull at the sites for placement of (1) the recording electrode: AP -3.5, ML +2.5, and DV(skull) -3.5 to -3.6, (2) the stimulating electrode: AP -4.0, ML +3.0, and DV(skull) -3.6 to -3.9, and (3) the ground electrode (roughly placed near the anterior-left corner of the operation area). The ground electrode was secured to the skull using a mini-screw, and the stimulating and recording electrodes were gently lowered until “just-before” their pre-defined brain region. The stimulating and the recording electrodes were optimally positioned in the stratum radiatum of the apical dendrites of the CA1 pyramidal neurons when the following characteristics were
observed: (1) an initial smaller EPSP in the apical dendrite, (2) evoked population spike and reversed EPSP in the cell body layer, and finally (3) a larger EPSC in the stratum radiatum of the apical dendrite. Thereafter, evoked population EPSP was recorded at a frequency of 30 seconds per stimulus, and allowed to equilibrate (typically took about 1 h). We then determined the input/output curve and the paired-pulse ratio, and began baseline recording at half-maximal response (ie. 500μA to get MAX -5mV, so used 110μA to get -2.5mV) with a pulse-width of 0.12msec and a negative polarity. This was usually within the range of 100-110μA, but sometimes up to 170μA.

The baseline EPSP response was measured for at least 60 min prior to induction of long term depression (LTD) of synaptic efficacy, and this was followed by another 2 h of EPSP recording. In selected animals, LTD was monitored for at least 6 h post-induction to confirm its long-lasting nature. Here, ischemic LTD (iLTD) was induced by global ischemia using methods described above (Chapter 2.2.2), and stimulus LTD (sLTD) was induced by low frequency stimulation (LFS) at 200 pulses x 0.5 Hz x paired-pulse depression (25msec interval) x 3 trains at 40min interval. Both protocols induced stable LTD that lasted for at least 2 h post-induction, and 6 h in all selected animals tested. At the end of recording, the input-output curve and the paired-pulse ratio were again measured. Selected animals subjected to iLTD were also recovered to confirm delayed neuronal death in CA1 region of hippocampus one week following global ischemia.

2.2.4 Perfusion and fixation

Each rat was anesthetized with urethane (1.5 g/kg, i.p.), and fixed by intra-cardiac perfusion as follows. The abdominal cavity of the rat was exposed via a v-shaped incision to the abdominal skin and the underlying abdominal muscles. The diaphragm was carefully
removed by a pair of scissors to expose the beating heart. The chest wall was deflected upwards by means of a large hemostat to facilitate operation of the heart. A small incision was made to the base of the cardiac ventricle, and the cannula for perfusion (while running saline) was placed into the left ventricle. The cannula was clamped in place with a small hemostat, and the right atrium was severed to allow circulation outflow. An incision to the neck and the underlying jugular vein was made to facilitate observation of the circulation outflow, which becomes largely cleared of blood after about 200 mLs of saline perfusion. The saline-line was thereafter switched off, and the paraformaldehyde-line switched on. Perfusion with the paraformaldehyde solution (4% paraformaldehyde in PBS) was characterized by muscle twitching, and ended when the twitching ceased and the rat became stiff (ie. fixed) throughout.

The head was bluntly displaced with a pair of scissors, and the skull and the overlaying skin were carefully peeled off with a pair of bone cutter designed for this purpose. The brain was collected in a 50-mL falcon tube filled with the paraformaldehyde solution, and kept in the 4°C fridge overnight. Thereafter, to achieve cryoprotection, the brain was kept for two days in 30% sucrose dissolved in the paraformaldehyde solution. The brain was then placed in the -80°C freezer for at least 20min, or for several days to weeks until it was ready to be sectioned by means of cryostat into 25-μm thick coronal sections. These coronal sections were then kept in PBS solution in the 4°C fridge, until they were needed for staining by Cresyl Violet (that stained cell nuclei), FluoroJade B (that stained degenerating neurons), immunohistochemistry for NeuN, or immunofluorescence for NR1 and SREBP1.
2.3 Human Embryonic Kidney Cells Model

2.3.1 Maintenance

Human embryonic kidney (HEK) 293 cell line were grown in a 10-cm Petri dish containing Dulbecco’s Modified Eagle Medium (D-MEM; Invitrogen, cat. #10566-016) with fetal bovine serum (FBS; Invitrogen, cat. #10099-141), stored in a 37°C incubator with 5% CO₂. They were monitored daily, and when density reached above 80%, they were divided and diluted into a new dish. Briefly, after removal of the old media from the Petri dish, and the HEK 293 cells were suspended by a 5-min incubation with 2mL of trypsin in D-MEM in the 37°C, 5% CO₂ incubator. The trypsin was then neutralized by 2mL of D-MEM/FBS, and the suspended HEK cells were collected by centrifugation at 1000 rpm for 10 min. Thereafter, the HEK cell-pellet was re-suspended in 4mL of fresh D-MEM/FBS by pipetting up-and-down, while avoiding air bubbles when mixing. Finally, 400µL of the newly suspended HEK cell line was diluted into a new Petri Dish containing 10mL of D-MEM/FBS to achieve a 1/10 dilution from the original culture. The diluted culture was then kept in the 37°C, 5% CO₂ incubator until used, or until it reached 80% density again.

2.3.2 Transfection and drug treatment

Transfection of plasmids into HEK cells was done using Lipofectamine (Invitrogen, cat. #15338-500) in accordance to the manufacturer’s instructions. Briefly, 4µg of DNA was diluted into 250µL of Opti-MEM (Invitrogen, cat. #31985-070), and 10µL of Lipofectamine was diluted into another 250µL of Opti-MEM. After 5-20 min of equilibration, the two Opti-MEM solutions were mixed and the final 500µL was allowed to further equilibrate for 20 min in room temperature. To initiate transfection, the 500µL DNA/Lipofectamine/Opti-MEM solution was added into each well of HEK cells ready to be transfected. The cells were then
kept in the 37°C incubator with 5% CO₂ for a 24-h transfection period, and the transfection media containing DNA/Lipofectamine/Opti-MEM was replaced with fresh D-MEM/FBS to terminate the transfection. In selected wells of transfected HEK cells, epoxomicin dissolved in PBS/DMSO solution (1mM epoxomicin in 10% DMSO in PBS) was diluted (1:1000 dilution to give 1µM epoxomicin) into the fresh D-MEM/FBS media to prevent proteasomal degradation. The control wells received either PBS/DMSO without epoxomicin, or no treatment. One day after drug/control treatment (or 48-h post-transfection onset), the cells were collected for western blot analysis.

2.3.3 Storage of HEK cells

Stocks of HEK cells were kept in liquid nitrogen using the following protocol. After making sure that the cellular density was sufficiently confluent (about 80%), the old D-MEM/FBS media was discarded by aspiration. The cells were suspended by 2mL of trypsin in D-MEM for 10 min at 37°C with gentle agitation, and following neutralizing the trypsin with 2mL of fresh DMEM/FBS, the cells were collected by centrifugation at 1000 rpm for 10 min. The HEK cell pellet was then resuspended in 9mL of FBS/DMSO solution (90% FBS and 10% DMSO), and one 1mL of which was pipetted into a freezer tube. The freezer tube was kept in -20°C for 1-2 h, in -80°C overnight or longer, and then stored in liquid nitrogen.

2.4 Tissue Collection and Western Blot Analysis

2.4.1 Animal tissue collection - whole cell lysate

To collect brain tissue for western blot analysis, each rat was euthanized by an overdose of urethane (2 g/kg, i.p.). The head was bluntly displaced with a pair of scissors, and the skull and the overlaying skin were carefully peeled off with a pair of bone cutter designed for this
purpose. The brain was gently removed, and coronally sectioned into six 2-mm-thick slices (from rostral to caudal, first to sixth). The M1 motor cortex was bluntly dissected from selected coronal sections, and homogenized in 500µL of 1% SDS solution, containing 1mM DTT, 1mM EDTA, 1mM EGTA, 1mM sodium vanadate, 10µg/ml Trasylol (Aprotinin), 10µg/ml Leupeptin, and 2mM PMSF (or 0.1M AEBSF) dissolved in PBS containing: 137mM NaCl, 2.7mM KCl, 4.3mM NA2HPO4, and 1.47mM KH2PO4 adjusted to pH 7.4. After about 30-strokes with the glass homogenizer, or until the tissue completely dispersed, the sample was cooked on a 100°C heat block for 5-10 min. Thereafter, the sample was left to cool down in room temperature. To crush any remaining cell aggregates, the sample was passed 6-times through a 25-Gauge syringe needle (with a 3mL syringe), and then left in room temperature for 20-50 min to allow the tissue to dis-integrate. The remaining tissue debris was removed by centrifugation at 14,000 rpm at room temperature. The soluble protein in the supernatant was collected and kept in -20°C freezer until use.

2.4.2 Animal tissue collection - nuclear fraction

To collect brain tissue for nuclear fraction and subsequent western blot analysis, each rat was euthanized to have the M1 motor cortex isolated from the brain as described above in Chapter 2.4.1. The isolated tissue was placed in a 1.5mL of pre-chilled Eppendorf tube containing 250µL of Buffer A, containing 10mM HEPES-KOH, 10mM KCl, 10mM EDTA, 1mM DTT, 0.4% NP40, the protease inhibitor cocktail (Roche, Complete-Mini; cat. #11836153001), and 1.5mM MgCl2 adjusted to pH 7.9. The tissue was cut into small pieces with a pair of surgical scissors, and homogenized by 15-20 strokes using a plastic homogenizer. The homogenized sample was allowed to sit on ice for at least 15 min prior to centrifugation at 850g for 10 min at 4°C. The supernatant was discarded, and another 200µL of Buffer A was added to further
homogenize the pellet by pipetting up-and-down. After 15 min of equilibration on ice, the sample was centrifuged at 14,000g for 3 min at 4°C, and the supernatant (or the cytosolic fraction) and pellet (or the crude nuclei) were separated into several Eppendorf tubes. The pellet fraction was further washed by mixing with another 200μL of Buffer A by pipetting up-and-down, and centrifuged at 14,000g for 3 min at 4°C to collect the pellet (clean nuclei). The pellet was cleaned again with 200μL of Buffer A (no dispersion this time), and then centrifuged at 14,000g for 3 min to collect the final pellet. The pellet containing the nuclei fraction was homogenized by pipetting up-and-down in 150μL of Buffer B, containing 20mM HEPES-KOH, 400mM NaCl, 1mM EDTA, 10% Glycerol, 1mM DTT, and the protease inhibitor cocktail (Roche, Complete-Mini; cat. #11836153001) adjusted to pH 7.9. The sample was further agitated at 150rpm on ice for 2 h, and centrifuged again at 15,000g for 5 min at 4°C. The soluble nuclear fraction in the supernatant was collected and kept in -20°C freezer until use.

2.4.3 HEK 293 cell tissue collection - whole cell lysate

To collect HEK cell line tissue for western blot analysis, the old D-MEM media in each Petri dish was replaced with 500μL of the radio-immuno-precipitation assay (RIPA) buffer containing: 150mM NaCl, 0.3% deoxycholic acid sodium, 50mM Tris, 1mM EDTA, 1.0% Triton X-100, 1% SDS, and the protease inhibitor cocktail (Roche, Complete-Mini; cat. #11836153001) at pH7.4. The cells were immediately scrapped off the Petri dish by specialized cell scraper, and cells suspended in RIPA were collected in an Eppendorf tube and immediately boiled on a 100°C heating block for 5 min. To further remove excessive cellular debris, the sample was passed 3-times through a 21-Gauge syringe needle, and another 3-times through a 25-Gauge syringe needle. The leftover debris after this was removed by
centrifugation at 14,000 rpm for 20 min, whereas the supernatant was kept in a clean Eppendorf tube stored in a -20°C freezer until use.

2.4.4 Electrophoresis and Western blot analysis

Proteins in the above mentioned tissue sample were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinyl difluoride (PVDF) membranes, blotted with specific primary and secondary antibodies, and detected by chemiluminescent imaging. First of all, 10µL of each the tissue sample was mixed by gentle agitation with 3.3µL of the sample buffer containing 50% glycerol, 125mM Tris-HCl (at pH 6.8), 4% SDS, 0.08% Bromophenol Blue, and 5% β-mercaptomethanol, and boiled on a 100°C heat block for 5 min. After cooled down, each sample was quickly centrifuged for 2 seconds, and loaded on to an SDS polyacrylamide gel immersed in running buffer containing 25mM Tris base, 190mM glycine, and 0.1% SDS at pH 8.3. A 8µL volume of protein ladder (ThermoScientific, PageRuler Prestained Protein Ladder; cat. #SM0671) was also loaded to each gel to facilitate protein identification. Here, SDS polyacrylamide gels (1.5mm-thick, with 10-wells) was prepared using standard cast (BIO-RAD, cat. #165-8000), and consisted of (1) a 7-mL 10% acrylamide separating gel made from 4.0mL of distilled water, 3.3mL of the 30% acrylamide solution, 2.5mL of 1.5M Tris (at pH 8.8), 100µL of 10% SDS, 50µL of 10% APS, and 5µL of TEMED, and (2) a 3-mL 5% acrylamide stacking gel made from 2.82mL of distilled water, 0.83mL of the 30% acrylamide solution, 1.25mL of 0.5M Tris (at pH 6.8), 50µL of 10% SDS, 50µL of 10% APS, and 5µL of TEMED. The gel was allowed to run for at least 3 h at 100V, and then each gel was secured onto a transfer chamber emerged in transfer solution composed of 25mM Tris base, 190mM glycine, and 20% methanol at pH 8.3. Thereafter, the proteins were wet-transferred at 100V for 90 min or 40V overnight, onto a
PVDF membrane for western blot analysis.

Western blot was performed by blocking the membrane for 2 h with 3% milk in PBS, followed by incubation with the primary antibody, prepared either in 3% milk in TBST for short term use or in 3% BSA in PBS for longer term storage in 4°C, for 2 h in room temperature or overnight in the 4°C cold room with constant agitation. Primary antibodies against insig1 (1:1000 dilution; synthesized *de novo* by Dr. Yu-Tian Wang Lab; rabbit polyclonal IgG), SREBP1 (1:1000 dilution; Santa Cruz; rabbit polyclonal IgG; cat. #sc-8984), GluR1 (1:250 dilution; Millipore; rabbit polyclonal IgG; cat. #05-855R), Prohibitin (1:200 dilution; Fitzgerald Industries; rabbit polyclonal IgG; cat. #RDI-PROHIBITab), insulin receptor α (1:1000 dilution; Santa Cruz; rabbit polyclonal IgG; cat. #sc-7953), Homer1b/c (1:3000 dilution; Santa Cruz; rabbit polyclonal IgG; cat. #sc-20807), GFP (1:8000 dilution; Clonetech; mouse monoclonal IgG2a; cat. #632381), β-tubulin (1:2000 dilution; Sigma; mouse monoclonal IgG1; cat. #T4026), β-actin (1:1000 dilution; Abcam; rabbit polyclonal IgG; cat. #ab8227), and NeuN (1:1000 dilution; Millipore; mouse monoclonal IgG1; cat. #MAB377) were used for western blot in the studies presented here. Following primary antibody, each membrane was washed 3 times with 5-10-min of TBST with agitation, and incubated in HRP-conjugated secondary antibodies specific for either mouse IgG or rabbit IgG for 1-2 h in room temperature. After another three 5-10-min washes with TBST, each membrane was detected by chemiluminescent imaging.

2.5 Histology, Immunohistochemistry, and Immunofluorescence

2.5.1 Cresyl violet staining

Paraformaldehyde-fixed coronal brain slices (25μm-thick) were mounted onto glass slides (three slices per glass slide), and allowed to dry in the dark overnight in room temperature.
On the day of Cresyl Violet staining, each glass slide was allowed to equilibrate in 100% ethanol for 3 min. Thereafter, it was sequentially hydrated by 3 min in 95% ethanol, 3 min in 70% ethanol, and 3 min in distilled water. Following the hydration steps, the glass slide was left to stain in 1% Cresyl Violet in distilled water for 10 min, and then rinsed twice with distilled water followed by sequentially de-hydration for 1 min in 70% ethanol, 1 min in 95% ethanol, and 1 min in 100% ethanol. Lastly, each glass slide was placed in 100% xylene solvent for at least 5 min to clear away ethanol, allowed to dry for a few seconds under a fume hood, and then mounted with Permount mounting media under a coverslip.

Image counting and analysis were done under a light microscope at 40X magnification. The number of viable cells (with round, healthy-looking nuclei), the number of cell debris (condensed particles) in the cellular layer, and the number of cell debris in the surrounding were counted within a 1mm^2 image frame taken from medial part of the CA1 and CA3 regions of the hippocampus. Image counting and analysis were done blinded by two or more experienced researchers, whose results were then averaged.

2.5.2 Fluoro-jade staining

Paraformaldehyde-fixed coronal brain slices (25µm-thick) were mounted onto glass slides (three slices per glass slide), and allowed to dry in the dark overnight in room temperature. On the day of Cresyl Violet staining, each glass slide was allowed to equilibrate in 100% ethanol for 3 min, and subsequently re-hydrated in 70% ethanol for 1 min and then distilled water for another 1 min. The slides were incubated in 0.06% potassium permanganate (KMnO4) for 15 min with agitation, and then rinsed in distilled water for 1 min. Each glass slide was stained by incubation for 30 min with gentle agitation in a 0.001% Fluoro-Jade B solution, prepared by diluting 20mL of a 0.01% stock solution (kept in 4°C until use) in
180mL of 0.1% acetic acid. After three 1-min washes in distilled water, each slide was allowed to dry in the dark overnight in room temperature. On the next day, the slides were cleared in three 2-min changes of 100% xylene solvent, allowed to dry for a few seconds under a fume hood, and then mounted with Permount mounting media under a coverslip.

Images of the whole hippocampus were taken using a fluorescent microscope, and area and intensity of Fluoro Jade staining was analyzed using Image J developed by the NIH. Area was expressed as a percentage ratio of the entire hippocampus. Intensity was expressed as the percentage ratio of CA1 versus dentate gyrus (DG; served as background control). The number of Fluoro Jade-positive neurons within a 1mm^2 image frame (taken from the medial part of the CA1) was counted at 40X magnification. Image counting and analysis were done blinded by two or more experienced researchers, whose results were then averaged.

2.5.3 Immunohistochemistry - NeuN

Immunohistochemistry was done in freely floating paraformaldehyde-fixed coronal brain slices (25μm-thick), and was facilitated by the use of 16-well plates for dividing solutions and brush for transferring between each well. Each brain slice was quenched of endogenous peroxidase by incubation with 3% hydrogen peroxide in distilled water, or with the ready-to-use peroxide blocking solution for 5 min (Chemicon, Anti-mouse poly HRP IHC detection kit; cat. #2764) in room temperature. After three 10-min washes in TBST containing 50mM Tris, 150mM NaCl and 0.05% Tween 20, it was incubated with the mouse IgG1 primary antibody for NeuN (Chemicon, Anti-NeuN clone A60; cat. #MAB377), prepared at 1:1000 dilution in 0.5% BSA in PBS, for 3 nights on a rocker platform for constant gentle agitation in a 4°C cold room. The PBS used here contained (mM): NaCl (137), KCl (2.7), NA2HPO4 (4.3), KH2PO4 (1.47) at pH 7.4. After another three 10-min washes in TBST, the brain slice was incubated
with the ready-to-use HRP-conjugated goat anti-mouse secondary antibody (Chemicon; cat. #2764) for 30 min in room temperature with gentle agitation. After yet another three 10-min washes in TBST, it was incubated with the freshly prepared DAB Chromogen-Buffer solution (Chemicon; cat. #2764), prepared by mixing the provided 25μL of DAB buffer with 1mL of DAB chromagen, for 5-20 min until the brown color developed. After five final 10-min washes with TBST, each brain slice was mounted onto a glass slide and allowed to dry in the dark overnight. The glass slide was then cleared with three 2-min incubation in 100% xylene solvent, mounted with Permount mounting media under a coverslip.

Image counting and analysis were done under a light microscope at 40X magnification. The number of NeuN+ cells were counted within a 1mm^2 image frame taken from medial part of the CA1 and CA3 regions of the hippocampus. Image counting and analysis were done blinded by two or more experienced researchers, whose results were then averaged.

2.5.4 Immunofluorescence - NR1 and SREBP1

Immunofluorescence was done in freely floating paraformaldehyde-fixed coronal brain slices (25μm-thick), and was facilitated by the use of 16-well plates for dividing solutions and brush for transferring between each well. Each brain slice was blocked for 30 min in room temperature with constant gentle agitation by a solution containing 1% bovine serum albumin (BSA) and 0.2% Triton X-100 in PBS, which contained (mM): NaCl (137), KCl (2.7), Na2HPO4 (4.3), KH2PO4 (1.47) at pH 7.4. After three 10-min rinses in PBS, it was incubated with the rabbit polyclonal IgG primary antibody for NR1 (Millipore; cat. #MAB363) prepared at 1:500 dilution in 0.5% BSA in PBS, or the rabbit polyclonal IgG primary antibody for SREBP1 (Santa Cruz, clone H-160; cat. #sc-8984) prepared at 1:100 dilution in 0.5% BSA in PBS, for 3 nights on a rocker platform for constant gentle agitation in
a 4°C cold room. After another three 10-min rinses in 0.5% BSA in PBS, the brain slice was incubated with the Alexa Fluor-555 goat anti-rabbit IgG (H+L) fluorescence-conjugated secondary antibody (Invitrogen; cat. #A-21428), prepared at 1:500 dilution in 0.5% BSA in PBS, for 2 nights with gentle agitation in a 4°C cold room. Bleaching was avoided at all times during and following incubation with the fluorescence-conjugated secondary antibody by wrapping up the 16-well plate with a piece of aluminum foil. After three final 10-min rinses in 0.5% BSA in PBS, each brain slice was mounted onto a glass slide and allowed to dry in the dark overnight. The brain tissue on the glass slide then received a drop of ProLong Gold antifade reagent with DAPI (Invitrogen; cat. #P-36935), and was mounted under a coverslip.

2.6 Molecular Biology and Genetic Engineering

2.6.1 Plasmid source

The mGFP-ATG-Homer1b pEGFP-c1 plasmid (origin: Rattus norvegicus (Norway rat)) (code: K600.0) was a kind gift from Drs. Yasunori Hayashi and Morgen Sheng at the Massachusetts Institute of Technology. The Homer1a-EGFP pEGFP-N plasmid (code: K300.0), the Homer1b-EGFP pEGFP-N plasmid (origin: Homo sapiens) (code: K400.0), and the mGlur5-EGFP pEGFP-N1 plasmid (code: K500.0) were kind gifts from Dr. Steve Furguson at the University of Toronto. The HA-Ubiquitin-R29,R48,R63 pcDNA3 plasmid (code: A200.0) and the pTAT plasmid (code: K900.0) were kind gifts from Drs. Pascal St-Pierre and Ivan Robert Nabi at UBC.

2.6.2 DNA amplification, purification, and storage

Upon receipt or modification, each plasmid was transformed, amplified, and purified by means of the Mini-Prep kit or the Maxi-Prep kit from QIAGEN (see List of Plasmids on Pages
Transformation was achieved by incubating 10μL of plasmid in 50μL of E. Coli DH5α competent cells for 30 min on ice, followed by a 45-seconds heat shock in a 37°C water bath. The transformed cells were immediately placed back on ice for another 2 min, and after supplying 250μL of LB Base, the cells were allowed to grow under agitation of 250 revolutions per min in a 37°C heat chamber for 40 min to 1 h. Thereafter, 150μL of transformed cells were distributed by means of a sterile glass rod (made sterile with a bunsen burner) onto a pre-heated agar plate (37°C), and then kept in a 37°C chamber overnight with each plate facing upside-down. On the next day, one culture colony was picked out of the agar plate with a sterile pipette tip, dipped into 5mL of LB Base containing the appropriate antibiotic (ampicillin or kanamycin), and allowed to grow under agitation (250 revolutions per min) in a 37°C heat chamber for 8 h (if not overnight) or 16-18 h (if overnight). This smaller amplified culture was either subjected to Mini-Prep purification kit (QIAGEN, QIAprep Spin Miniprep Kit; cat. #27106) in accordance to the manufacturer’s instructions, or subjected to a larger amplification in a 300μL LB Base with antibiotic overnight (12-16 h) in the same manner. The larger amplified culture was then purified using the Maxi-Prep purification kit (QIAGEN, QIAfilter Plasmid Maxi Kit; cat. #12263) in accordance to the manufacturer’s instructions. After plasmid verification by gel electrophoresis and/or IDT DNA sequencing, each plasmid was coded and stored under -20°C until needed.

2.6.3 Site directed mutagenesis

Site directed mutagenesis was achieved by performing poly-chain reactions (PCR) with the aid of the QuikChange Site-Directed Mutagenesis Kit (Stratagene; cat. #200518) in accordance to the manufacturer’s instructions but with some modifications. Each PCR sample contained 1.0μL of QuikChange lightning enzyme (which was added last), 10-100ng of the input
template, 1.25μL of the sense primer (100 ng/μL), 1.25μL of the anti-sense primer (100 ng/μL), 1μL of deoxy-nucleotide tri-phosphate (dNTP) solution, 5μL of 10x reaction buffer, 1.5μL QuikSolution reagent, and made up to 50μL with distilled water. The PCR reaction was ran by an automated thermo-cycler set to run 18 repeated cycles of induction: 95˚C for 25 seconds, annealing: 60˚C for 10 seconds, and elongation: 68˚C for 3 min, and after the repeated cycles, the PCR reaction was terminated by 68˚C for 5 min and kept in 4˚C until sample was collected. The parent-DNA was then digested by 2μL of Dpn I restriction enzyme, mixed with the 50μL PCR sample by pipetting up-and-down, for 5 min in 37˚C water bath. About 2μL of the final sample was needed for transformation, amplification, and purification using the above-mentioned methods (see Chapter 2.4.2).

2.6.4 Restriction and ligation
Restrictions of plasmid DNA to allow insertions of additional DNA sequences were done using enzymes specific for the EcoRI (cat. #B0101S), the NheI (cat. #R0131L), and the HindIII (cat. #R0104L) restriction sites, purchased from New England BioLabs. Each reaction solution contained 5μg of each plasmid, 50 Units of each restriction enzyme, 0.5μL of 100X BSA (10 mg/kg stock), 5μL of the provided 10x NEBuffer, and made up to a total of 50μL with distilled water. The enzymes were allowed to react for 2 h in a 37˚C water bath, and were then heat-inactivated by 65˚C for 20 min in the PCR thermal cycler. This procedure consistently produced successful restriction in our preparation. To induce re-ligation, 15.5μL of the above reaction solution was further mixed with 0.5μL of 1Unit/μL T4 Ligase (Invitrogen) and 4μL of the provided 5X ligation buffer. The ligation reaction normally took at least 1 h in room temperature (23˚C), and sometimes overnight. Finally, the ligated DNA was diluted 5X in distilled water (10μL of ligation solution + 40μL of distilled water) prior to
transformation, amplification, and DNA purification (See Chapter 2.6.2). Notably, the last dilution step substantially enhanced transformation efficiency.

2.6.5 Plasmid production

The poly-ubiquitination sites on the A200.0 HA-Ubiquitin-R29,R48,R63 plasmid were recovered by site-directed mutagenesis to obtain the A201.0 HA-Ubiquitin-K29,R48,R63, the A201.1 HA-Ubiquitin-R29,K48,R63, the A201.2 HA-Ubiquitin-R29,R48,K63, and the A201.3 HA-Ubiquitin-R29,R48,R63-G76V plasmids using the following primer pairs:

R29K sense: 5’-GAA AAT GTA AAG GCC AAG ATC CAG GAT AAG GAA GG-3’
R29K antisense: 5’-CCT TCC TTA TCC TGG ATC TTG GCC TTT ACA TTG TC-3’

R48K sense: 5’-CTG ATC TTT GCT GGC AAG CAG CTG GAA GAT GGA CG-3’
R48K antisense: 5’-CGT CCA TCT TCC AGC TGC TTG CCA GCA AAG ATC AG-3’

R63K sense: 5’-GAC TAC AAT ATT CAA AAG GAG TCT ACT CTT CAT C-3’
R63K antisense: 5’-GAT GAA GAG TAG ACT CCT TTT GAA TAT TGT AGT C-3’

G76V sense: 5’-GTT GAG ACT TCG TGG TGT TGT ACT CGA GCA TGC ATC TAG-3’
G76V antisense: 5’-CTA GAT GCA TGC TCG AGT AGT ACA ACA CCA CGA AGT CTC AAC-3’

These newly generated plasmids were further mutated into the A202.1 HA-Ubiquitin-R29, the A202.2 HA-Ubiquitin-R48, and the A202.3 HA-Ubiquitin-R63 plasmids using the same primer pairs above. Finally, the A202.1 HA-Ubiquitin-R29 plasmid was mutated into the A203.0 HA-Ubiquitin plasmid encoding wild-type ubiquitin using the R29K primer pairs described
above.

To facilitate the insertion of these ubiquitin plasmids into other plasmids for the expression of linear ubiquitinated-proteins, the STOP codon was converted into an EcoRI restriction site to generate the A204.0 HA-Ubiquitin(no-STOP)-EcoRI, the A204.1 HA-Ubiquitin-R29(no-STOP)-EcoRI, the A204.2 HA-Ubiquitin-R48(no-STOP)-EcoRI, the A204.3 HA-Ubiquitin-R63(no-STOP)-EcoRI, and the A204.4 HA-Ubiquitin-R29,R48,R63(no-STOP)-EcoRI plasmids using the following pair of primers:

EcoRI end sense: 5’-G TTG AGA CTT CGT GGT GGT GAA TTC GAG CAT GCA TCT AG-3’
EcoRI end antisense: 5’-CT AGA TGC ATG CTC GAA TTC ACC ACC ACG AAG TCT CAA C-3’

To confer resistance to de-ubiquitinating enzymes (DUB), the above mentioned ubiquitin plasmids were further modified to have the last amino-acid-residue mutated from glycine into valine, generating the A204.5 HA-Ubiquitin(G76V)-EcoRI, the A204.6 HA-Ubiquitin-R29(G76V)-EcoRI, the A024.7 HA-Ubiquitin-R48(G76V)-EcoRI, and the A204.8 HA-Ubiquitin-R29,R48,R63(G76V)-EcoRI plasmids, using the following primers:

G76V-EcoRI end sense: 5’-G TTG AGA CTT CGT GGT GTT GAA TTC GAG CAT GCA TCT AG-3’
G76V-EcoRI end antisense: 5’-CT AGA TGC ATG CTC GAA TTC ACC ACC ACG AAG TCT CAA C-3’

The extra EcoRI site on the 5’-terminus of these ubiquitin plasmids were removed, leaving only NheI site as the only restriction site between DNA encoding HA and ubiquitin, to generate the A205.0 NheI-Ubiquitin-EcoRI, the A205.1 NheI-Ubiquitin-R29-EcoRI, the A205.2 NheI-Ubiquitin-R48-EcoRI, the A205.3 NheI-Ubiquitin-R-63-EcoRI, the A205.4 NheI-Ubiquitin(G76V)-EcoRI, the A205.5 NheI-Ubiquitin-R29(G76V)-EcoRI, the A205.6 NheI-
Ubiquitin-R48(G76V)-EcoRI, and the A205.7 Nhel-Ubiquitin-R63(G76V)-EcoRI plasmids using the following primers:

EcoRIgone sense: 5’-GCT AGC CTC GGT AAA TTC ATG CAG-3’
EcoRIgone antisense: 5’-CTG CAT GAA TTT ACC GAG GCT AGC-3’

The K400.0 Homer1b-EGFP pEGFP plasmid was mutated to become the K401.0 Homer1b-mGFP plasmid to avoid inter-GFP interaction following expression in HEK cells, using the following primers (originally designed by Roger Tsien):

A206K sense: 5’-CAG TCC AAG CTG AGC AAA GAC CCC AAC GAG AAG CGC GAT CAC-3’
A206K antisense: 5’-GTG ATC GCG CTT CTC GTT GGG GTC TTT GCT CAG CTT GGA CTG-3’

and this K401.0 plasmid was subsequently mutated to include an EcoRI site in its 5’-terminus, generating the K402.0 EcoRI-Homer1b-mGFP plasmid using the following primers:

EcoRI-H1b sense: 5’-AGA TCC GCT AGC AAA ATG GGG GAA CAAC CCT ATC TTC AGC ACT GAG GCT CAT -3’
EcoRI-H1b antisense: 5’-ATG AGC TCG AGT GCT GAA GAT AGG TTG TTC CCC CAT GAA TTC GCT AGC GGA TCT-3’

This allowed insertion of the Ubiquitin sequence from the K205.4 Nhel-Ubiquitin(G76V)-EcoRI, the K204.5 HA-Ubiquitin(G76V)-EcoRI, and the K204.0 HA-Ubiquitin(no-STOP)-EcoRI plasmid into the new K402.0 EcoRI-Homer1b-mGFP plasmid to generate the K402.1 Nhel-Ubiquitin(G76V)-ATG-Homer1b-mGFP, the K402.2 EcoRI-ATG-Ubiquitin(G76V)-
ATG-Homer1b-mGFP, the K402.3 EcoRI-Ubiquitin(GG)-ATG-Homer1b-mGFP plasmids, respectively, by means of NheI/EcoRI double-restriction or EcoRI restrictions, followed by ligation. Finally, the start codon on Homer1b sequence of the K402.1 NheI-Ubiquitin(G76V)-ATG-Homer1b-mGFP plasmid was removed to produce the K402.4 Ubiquitin(G76V)-Homer1b-mGFP plasmid used for transfection, using the following primers:

UTnoATG sense: 5’-CGT GGT GTT GAA TTC ATC GGG GAA CAA CCT ATC TTC AGC-3’
UTnoATG antisense: 5’-GCT GAA GTA AGG TTG TTC CCC GAT GAA TTC AAC ACC ACG-3’

To truncate the mGFP out of the expressed protein, the K402.0 EcoRI-Homer1b-mGFP, the K402.1 NheI-Ubiquitin(G76V)-ATG-Homer1b-mGFP, and the K402.4 Ubiquitin(G76V)-Homer1b-mGFP plasmid was mutated to include a de novo STOP codon in the 3’-terminus of Homer1b to generate the K403.2 EcoRI-Homer1b-TAGstop, K403.0 Ubiquitin(G76V)-ATG-Homer1b-TAGstop and the K403.1 Ubiquitin(G76V)-Homer1b-TAGstop plasmids, respectively, using the following primers:

XSTOPX sense: 5’-GAA TGC AGC TAG CGG GCC CGG GTC GCC ACC-3’
XSTOPX antisense: 5’-GGT GGC GAC CCG GGC CCG TTA GCT GCA TTC-3’

Similarly, the K402.4 Ubiquitin(G76V)-Homer1b-mGFP plasmid was mutated to generate the K403.3 Ubiquitin(G76V)-Homer1b-TAAstop plasmid with the following primer pair:

XSTOPXXTAA sense: 5’-GAA TGC AGC TAA CGG GCC CGG GTC GCC ACC-3’
XSTOPXXTAA antisense: 5’-GGT GGC GAC CCG GCC CCG TTA GCT GCA TTC-3’
and this new plasmid was further mutated to generate the K404.0 *Ubiquitin(G76V)-Homer1b-Nterminus* and the K404.1 *Ubiquitin(G76V)-Homer1b-Cterminus* plasmids used for transfection, using the following primer pairs:

**H1b-N sense:** 5’-CA AAG GAA AAA TCA CAA GAG TAG ATG GAA CTT ACC AGT ACA CC-3’

**H1b-N antisense:** 5’-GG TGT ACT GGT AAG TTC CAT CTA CTC TTG TGA TTT TTC CTT CTG TG-3’

**H1b-C sense:** 5’-GC AAA CAT TGG GAG GCT GAA TTC CTC CCA ATG TTT GC-3’

**H1b-C antisense:** 5’-CC TTT GAG GGT AGC GAA TTC AGC CTC CCA ATG TTT GC-3’

The K403.3 *Ubiquitin(G76V)-Homer1b-TAAstop* plasmid also served as host for the insertion of the various *Ubiquitin* sequence from the above mentioned plasmids, in the generation of the K403.4 *Ubiquitin(G76V)-Homer1b-TAAstop*, the K405.3 *Ubiquitin-R29(G76V)-Homer1b-TAAstop*, the K405.5 *Ubiquitin-R48(G76V)-Homer1b-TAAstop*, the K405.6 *Ubiquitin-R63(G76V)-Homer1b-TAAstop*, and the K405.7 *Ubiquitin-R29,R48(G76V)-Homer1b-TAAstop* plasmids by means of Nhel/EcoRI double-restrictions and re-ligation. The K405.0 *Ubiquitin(G76V)-R6-Homer1b-TAAstop*, the K405.1 *Ubiquitin-R11(G76V)-Homer1b-TAAstop*, the K405.2 *Ubiquitin-R27(G76V)-Homer1b-TAAstop*, the K405.4 *Ubiquitin-R33(G76V)-Homer1b-TAAstop*, the K405.8 *Ubiquitin-Q29(G76V)-Homer1b-TAAstop* plasmid, and the K405.9 *Ubiquitin-Q48(G76V)-Homer1b-TAAstop* plasmids were also generated from the K403.3 *Ubiquitin(G76V)-Homer1b-TAAstop* plasmid by site-directed mutagenesis using the following primers:

**R6 sense:** 5’-CAG ATT TTC GTG AGA ACC CTT ACG GGG AAG ACC ATC ACC-3’

**R6 antisense:** 5’-GGT GAT GGT CTT CCC CGT AAG GGT TCT CAC GAA AAT CTG-3’
R11 sense: 5’-GTG AAA ACC CTT ACG GGG AGG ACC ATC ACC CTC-3’
R11 antisense: 5’-GAG GGT GAT GGT CCT CCC CGT AAG GGT TTT CAC-3’

R27 sense: 5’-CCC TCG GAT ACG ATA GAA AAT GTA AGG GCC AAG ATC CAG GAT AAG-3’
R27 antisense: 5’-CTT ATC CTG GAT CTG CCT TAC ATT TTC TAT CGT ATC CGA GGG-3’

R33 sense: 5’-G GCC AAG ATC CAG GAT AGG GAA GGA ATA CCT CC-3’
R33 antisense: 5’-GG AGG TAT TCC TTC CCT ATC CTG GAT CTT GGC C-3’

R29 sense: 5’-CG ATA GAA AAT GTA AAG GCC AGG ATC CAG GAT AAG GAA GG-3’
R29 antisense: 5’-CC TTC CTT ATC CTG GAT CTG GGC CTT TAC ATT TTC TAT CG-3’

Q29 sense: 5’-CG ATA GAA AAT GTA AAG GCC CAG ATC CAG GAT AAG GAA GG-3’
Q29 antisense: 5’-CC TTC CTT ATC CTG GAT CTG GGC CTT TAC ATT TTC TAT CG-3’

Q48 sense: 5’-CTG ATC TTT GCT GGC CAG CAG CTG GAA GAT GGA CG-3’
Q48 antisense: 5’-CG TCC ATC TTC CAG CTG CTG GCC AGC AAA GAT CAG-3’

To express N-terminal truncations of ubiquitinated-Homer1b (or ubiquitin-fragment-linked Homer1b), the START codon on the K403.3 Ubiquitin(G76V)-Homer1b-TAAstop plasmid was first removed by site-directed mutagenesis to generate the K406.0 Xbiquitin(G76V)-Homer1b-TAAstop using the following primers:

Xbi sense: 5’-GCT AGC CTC GGT AAA TTC ATC CAG ATT TTC GtG AAA ACC C-3’
Xbi antisense: 5’-G GGT TTT CAC GAA AAT CTG GAT GAA TTT ACC GAG GCT AGC-3’

and this plasmid in turn was used as a host to generate the K406.1 Xbiquitin(G76ATG)-
Homer1b, the K406.2 Ubi(10-76V)-Homer1b, the K406.3 Ubi(21-76V)-Homer1b, the K406.4 Ubi(30-76V)-Homer1b, the K406.5 Ubi(37-76V)-Homer1b, the K406.6 Ubi(49-76V)-Homer1b, and the K406.7 Ubi(64-76V)-Homer1b plasmids by site-directed mutagenesis using the following primers:

Xbi-ATG sense: 5’-GTG TTG AGA CTT CGT GGT ATG GAA TTC ATC GGG GAA CAA CC-3’
Xbi-ATG antisense: 5’-GG TTG TTC CCC GAT GAA TTC C AT ACC ACG AAG TCT CAA CAC-3’

START10 sense: 5’-GTG AAA ACC CTT ATG GGG AAG ACC ATC ACC CTC-3’
START10 antisense: 5’-GAG GGT GAT GGT CTT CCC CAT AAG GGT TTT CAC-3’

START21 sense: 5’-CC ATC ACC CTC GAA GTT GAA CCC ATG GAT ACG ATA GAA AAT G-3’
START21 antisense: 5’-C ATT TTC TAT CGT ATC CAT GGG TTC AAC TTC GAG GGT GAT GG-3’

START30 sense: 5’-CG ATA GAA AAT GTA AAG GCC ATG ATC CAG GAT AAG GAA GG-3’
START30 antisense: 5’-CC TTC CTT ATC CTG GAT CAT GGC CTT TAC ATT TTC TAT CG-3’

START37 sense: 5’-CAG GAT AAG GAA GGA ATG CCT CCT GAT CAG CAG AGA CTG-3’
START37 antisense: 5’-CAG TCT CTG CTG ATC AGG AGG CAT TCC TTC CTG ATC CTG-3’

START49 sense: 5’-CTG ATC TTT GCT GGC ATG CAG CTG GAA GAT GG-3’
START49 antisense: 5’-CC ATC TTC CAG CTG CAT GCC AGC AAA GAT CAG-3’

START64 sense: 5’-G TCT GAC TAC AAT ATT CAA ATG GAG TCT ACT CTT CAT GTG-3’
START64 antisense: 5’-CAC AAG ATG AAG AGT AGA CTC CAT TTG AAT ATT GTA GTC AGA C-3’

To make C-terminus truncation of ubiquitinated-Homer1b, the K403.3 Ubiquitin(G76V)-
*Homer1b-TAA*<sup>stop</sup> plasmid was mutated to include the EcoRI site in various positions, followed by EcoRI restriction and ligation to generate the K407.0 *Ubi(1-57)-Homer1b*, the K407.1 *Ubi(1-45)-Homer1b*, and the K407.2 *Ubi(1-38)-Homer1b* plasmids. The following primers were used:

38-EcoRI sense: 5’-G GAA GGA ATA CCT CCT GAA TTC CAG AGA CTG ATC TTT GCT GGC AAG CAG C-3’  
38-EcoRI antisense: 5’-G CTG CTT GCC AGC AAA GAT CAG TCT CTG GAA TTC AGG AGG TAT TCC TTC C-3’

45-EcoRI sense: 5’-CCT GAT CAG CAG AGA CTG ATC TTT GAA TTC AAG CAG CTG GAA GAT GGA CG-3’  
45-EcoRI antisense: 5’-CG TCC ATC TTC CAG CTG CTT GAA TTC AAA GAT CAG TCT CTG CTG ATC AGG-3’

57-EcoRI sense: 5’-CAG CTG GAA GAT GGA CGT ACT TTG TCT GAA TTC AAT ATT CAA AAG GAG TC-3’  
57-EcoRI antisense: 5’-GA CTC CTT TTG AAT ATT GAA TTC AGA CAA AGT ACG TCC ATC TTC CAG CTG-3’

These primers were also used to generate plasmids encoding short-ubiquitin-fragment-linked Homer1b, including the K408.0 *Ubi(37-45)-Homer1b*, the K408.1 *Ubi(37-57)-Homer1b*, the K408.2 *Ubi(10-45)-Homer1b*, the K408.3 *Ubi(10-38)-Homer1b*, and the K408.4 *Ubi(21-38)-Homer1b* plasmids using site-directed mutagenesis from the above mentioned plasmids encoding N-terminus truncation of ubiquitinated-Homer1b.

Plasmids encoding carboxyl-terminus of the mGluR5 (mGluR5CT) were generated from the K500.0 *mGluR5-EGFP* pEGFP-N1 plasmid. First, an EcoRI site was inserted in the
mGluR5 sequence using the following primers:

EcoRI-mCT sense: 5’-GAA GGG CGA TGG TGG AGT GAA TTC CAC AAG CTC CTC CAG ATC-3’
EcoRI-mCT antisense: 5’-GAT CTG GAG GAG CTT GTG GAA TTC ACT CCA CCA TCG CCC TTC-3’

and following EcoRI restriction and ligation, resulted in the K502.0 pre-mCT-EGFP plasmid, which in turn was used to generate (1) the K502.1 ATG-mCT-EGFP plasmid by inserting Xbiquitin(ATEGFP) from the K406.1 Xbiquitin(G76ATG)-Homer1b plasmid, and (2) the K502.2 Ubi(G76V)-mCT-EGFP by inserting Ubiquitin(G76V) from the K403.3 Ubiquitin(G76V)-Homer1b-TAAstop plasmid, both via NheI/EcoRI double-restriction and re-ligation. The K502.1 and the K502.2 plasmids were further used to generate the K502.3 ATG-mCT(P>Q)-EGFP, the K502.4 Ubi(G76V)-mCT(P>Q)-EGFP, the K502.5 Ubi(G76V)-mCT8, the K502.6 Ubi(G76V)-mCT8(P>Q), and the K602.7 Ubi(G76V)-mCT8(F>R) plasmids using the following primers:

mCT8 sense: 5’-GGT GCT CCC CGA GTC CTA CGA GTC CCT GAA GGG-3’
mCT8 antisense: 5’-CCC TTC AGG GAC TCG TAG GAC TCG GGG AGC ACC-3’

mCT(P>Q) sense: 5’-GAA TTC ACT CCA CAA TCG CCC TTC AGG GAC TCG-3’
mCT(P>Q) antisense: 5’-CGA GTC CCT GAA GGG CGA TTG TGG AGT GAA TTC-3’

mCT(F>R) sense: 5’-CCA CCA TCG CCC CGC AGG GAC TCG TAG GAC TCG GGG-3’
mCT(F>R) antisense: 5’-CCC CGA GTC CTA CGA GTC CCT GCG GGG CGA TGG TGG-3’

A STOP codon and a HindIII restriction site was placed at the 3’-terminus of the Homer1b in these above plasmid by means of site-directed mutagenesis, thus essentially
removed the mGFP-link from the expressed protein. This resulted in the generation of the K503.0 \textit{ATG-mCT}, the K503.1 \textit{Ubi(G76V)-mCT}, the K503.2 \textit{ATG-mCT(P>Q)}, the K503.3 \textit{Ubi(G76V)-mCT(P>Q)}, the K503.4 \textit{Ubi(G76V)-mCT8-HindIII}, and the K503.5 \textit{Ubi(G76V)-mCT8(P>Q)-HindIII} plasmids with the following primers:

mCT-HindIII sense: 5’-GT TCT TCA TCG TTG TAG AAG CTT ACG GTA CCG CGG GCC CGG GAT CCA CCG G-3’
mCT-HindIII antisense: 5’-C CGG TGG ATC CCG GGC CCG CGG TAC CGT AAG CTT CTA CAA CGA TGA AGA AC-3’

The K600.0 \textit{mGFP-ATG-Homer1b} pmGFP-C1 plasmid was used to generate the K600.3 \textit{mGFP} by including a \textit{de novo} STOP codon behind the \textit{mGFP} sequence, and the K600.1 \textit{mGFP-EcoRI-ATG-Homer1b} plasmid by inserting the \textit{EcoRI} restriction site right before the \textit{Homer1b} sequence. Moreover, this K600.1 plasmid was in turn used to generate the K600.2 \textit{mGFP-Homer1b} plasmid by removing the START codon. The following primers were used for site-directed mutagenesis described here:

MH-EcoRI sense: 5’-CAA GCT TCC GAA TTC ATG GGG GAA C-3’
MH-EcoRI antisense: 5’-G TTC CCC CAT GAA TTC GGA AGC TTG-3’

MHnoSTART sense: 5’-GCT CAA GCT TCC GAA TTC ATC GGG GAA CAA CCT ATC-3’
MHnoSTART antisense: 5’-GAT AGG TTG TTC CCC GAT GAA TTC GGA AGC TTG AGC-3’

M-STOP sense: 5’-C GAG CTG TAC AAG TCC T GAC TCA GAT CTC GAG C-3’
M-STOP antisense: 5’-G CTC GAG ATC TGA GTC A GGA CTT GTA CAG CTC G-3’
The K600.1 mGFP-\textit{EcoRI-ATG-Homer1b} plasmid also served as a host to generate high-expression plasmids encoding ubiquitinated-Homer1b (with \textit{Rattus norvegicus} (Norway rat) version of Homer1b), including the K601.0 \textit{NheI-Ubiquitin-\textit{EcoRI-Homer1b(rat)}}, the K601.1 \textit{NheI-Ubiquitin(G76V)-\textit{EcoRI-Homer1b(rat)}}, the K601.2 \textit{NheI-Ubiquitin-R29(G76V)-\textit{EcoRI-Homer1b(rat)}}, the K601.3 \textit{NheI-Ubiquitin-R48(G76V)-\textit{EcoRI-Homer1b(rat)}}, and the K601.4 \textit{NheI-Ubiquitin-R63(G76V)-\textit{EcoRI-Homer1b(rat)}} plasmids by inserting the wild-type and mutant \textit{Ubiquitin} sequences from the above mentioned plasmids, by means of NheI/EcoRI double-restriction and re-ligation.
CHAPTER 3:
SYNAPTIC/NR2A NMDAR STIMULATION

3.1 Specific Background

3.1.1 NMDAR electrophysiology and pharmacology

The NMDAR is a type of cation-permeable ionotropic receptor that mediates fast excitatory synaptic transmission by glutamate, the primary excitatory neurotransmitter in the mammalian central nervous system (Cull-Candy et al., 2001). NMDAR subunits are classified into three subfamilies: NR1, NR2 (A-D) and NR3 (A and B) (Flint et al., 1997; Kirson and Yaari, 1996; Li et al., 1998; Stephenson, 2001; Stocca and Vicini, 1998), and native NMDARs are tetrameric complexes comprised of two obligatory NR1 subunits coupled to at least one NR2 subunit (Flint et al., 1997; Kirson and Yaari, 1996; Laube et al., 1998; Li et al., 1998; Schorge and Colquhoun, 2003; Stephenson, 2001; Stocca and Vicini, 1998; Ulbrich and Isacoff, 2007). These receptors differ from other ionotropic glutamate receptors in that they conduct calcium in addition to sodium and potassium (Berdichevsky et al., 1983; MacDermott et al., 1986). Moreover, they are inwardly rectified due to channel gating by extracellular magnesium ions (Ault et al., 1980; Mayer et al., 1984; Nowak et al., 1984); therefore, channel opening requires simultaneous membrane depolarization and agonists-binding. In addition, binding of both NMDAR co-agonists, (1) glycine to the NR1 subunits, and (2) glutamate to the NR2 subunits is required for channel activation (Johnson and Ascher, 1987; Kleckner and DINGledine, 1988; Lerma et al., 1990). Therefore, glutamate only activates NMDAR at glycine’s presence, and increasing glycine concentration only increases NMDAR activity at glutamate’s presence.
Likewise, selective competitive antagonists that bind to either the glutamate-binding site or the glycine-binding site can sufficiently antagonize the NMDAR channel function completely (Johnson and Ascher, 1987; Kleckner and Dingerdine, 1988; Lerma et al., 1990).

3.1.2 Stimulating pro-survival NMDARs

As discussed in Chapter 1.3, the NMDAR location and subtype hypotheses are not entirely contradictory, because in the adult forebrain, where stroke most frequently occurs, the synapses are populated by NR2A-containing NMDARs (NR2ARs) and the extrasynaptic sites are populated by NR2B-containing NMDARs (NR2BRs) (Cull-Candy et al., 2001; Tovar and Westbrook, 1999). Therefore, stimulating synaptic NMDARs would be stimulating mostly NR2ARs, resulting in increased neuronal survival. Likewise, blocking extrasynaptic NMDARs would be blocking most of the NR2BRs, and vice versa, resulting in neuroprotection against stroke damage. One drug that can selectively activate synaptic NMDARs is the NMDAR co-agonist glycine. Glutamate is normally found at the synapses where it is released from the presynaptic terminals. Following activation of the postsynaptic synaptic/NR2A NMDARs, it is quickly taken up by glutamate transporters before reaching the extrasynaptic sites. Given the restricted localization of glutamate to the synapses, the co-agonist glycine will only activate synaptic (mostly NR2A) NMDARs, resulting in increased neuronal survival. Note that, even though glutamate is released to the extrasynaptic sites during stroke (Kanthan et al., 1995; Rossi et al., 2000), the elevation of extracellular glutamate concentration is short-lived and quickly recovers back to normal a few hours after stroke (Kanthan et al., 1995).
3.2 Results

3.2.1 NMDAR subtypes on stroke damage

Conventional NMDAR antagonists tested in stroke trials are not subtype-selective against NR2ARs and NR2BRs, and based on the NMDAR subtype and location hypothesis, their neuroprotective effect primarily comes from inhibiting extrasynaptic/NR2B NMDARs (Lai et al., 2011). This suggests that NR2BR antagonists are sufficiently neuroprotective, whereas NR2AR antagonists are not neuroprotective. To determine whether native NR2ARs and NR2BRs have opposing roles in stroke outcome, we injected rats with subtype-selective antagonists NVP-AAM007 (2.4 mg/kg, i.v.) for NR2ARs or Ro 25-6981 (6 mg/kg, i.v.) for NR2BRs 30 min before subjecting them to MCAO (FIGURE 3-1). The doses for these two compounds used in the present study have been previously shown to selectively inhibit NMDAR-subtype-specific synaptic plasticity in vivo (Fox et al., 2006). As predicted, selective inhibition of NR2BRs with Ro 25-6981 was strongly neuroprotective. In contrast, selective inhibition of NR2ARs with NVP-AAM077 did not provide neuroprotection; instead, it increased cerebral infarction following stroke. This suggested that NR2AR has an intrinsic neuroprotective role during stroke, and its inhibition can be detrimental to stroke outcome. Moreover, this could explain why some NMDAR blockers appeared to have exacerbated stroke outcome in clinical trials (Albers et al., 2001; Lees et al., 2001). Importantly, these results raised the intriguing possibility that stimulation of NR2ARs could confer neuroprotection by potentiating this intrinsic neuroprotective pathway.

3.2.2 Glycine treatment: neuroprotection against focal ischemic stroke

As described in chapter 3.1.2, one compound that can selectively stimulate the pro-survival NMDARs in the synapse is the NMDAR co-agonist glycine, because its effect on NMDAR
requires the simultaneous presence of glutamate which is normally only in the synapse. Although glutamate becomes elevated during the onset of stroke, the increase is only transient and quickly recovers to baseline level following reperfusion of blood to the brain. To test the hypothesis that stimulation of pro-survival NMDARs can confer neuroprotection against stroke, we injected rats subjected to MCAO with glycine (800 mg/kg, i.p.) at 4.5 h post-ischemia onset (FIGURES 3-2 and 3-3). This dose of glycine was selected based on a previous study showing its potentiation of NMDAR-dependent behavior in vivo (De Sarro et al., 2000). Consistent with our hypothesis, glycine improved 24-h stroke outcome in neurological tests that examined motor and sensory capacity, including the left/right motor symmetry test, the tactile side test, the visual side test, and the proprioceptive test, but had no effect in tests that examined motor and sensory preference, such as the visual forward test and the tactile forward test (FIGURE 3-2).

One main reason that NMDAR blockers failed clinical studies was that, once the downstream death signals are activated shortly after stroke, these receptor antagonists becomes no longer effective (see Chapter 1.6 for thorough discussion). In developing novel NMDAR-based therapeutics, we hypothesized that drugs that act by stimulating the NR2AR survival signaling pathway do not need to be administered prior to the activation of death signaling proteins downstream of NR2BRs, and thereby can confer a wider therapeutic window compared to NMDAR blockers. Consistent with earlier reports by others (Endres et al., 1998), the conventional NMDAR blocker MK-801 (1mg/kg, i.p.) failed to protect the rat brain against MCAO-induced cerebral infarction when given at a delayed time point of 4.5 h post-ischemia onset (FIGURE 3-3). In marked contrast, our proposed strategy of stimulating pro-survival NMDARs with glycine is strongly neuroprotective when given at this delayed time point. In line with the notion that glycine confer neuroprotection by stimulating pro-
survival NMDARs, glycine failed to protect the brain against cerebral infarction in the presence of MK-801 (FIGURE 3-3). Moreover, the neuroprotective effect of glycine was not blocked by the NR2BR blocker Ro 25-6981, but was blocked by the NR2AR blocker NVP-AAM077, suggesting the effect is mediated through the stimulation of synaptic/NR2ARs (FIGURE 3-4). Notably, this later experiment does not presume whether glycine mediates neuroprotection by stimulating NR2AR per se or by stimulating synaptic NMDARs per se, because in the adult forebrain, most of the NR2ARs are in the synapse and inhibiting NR2ARs with NVP-AAM077 would be inhibiting most of the synaptic NMDARs. In any case, our study lands strong evidence that stimulating pro-survival NMDARs, be it synaptic NMDARs or NR2ARs, is neuroprotective against stroke damage.

In consideration of the potential wide therapeutic time window for neuroprotection by stimulating NMDAR survival pathway, we sought to test if glycine is neuroprotective when the treatment is delayed up to 6 h post-ischemia onset (FIGURE 3-5). To rule out any inadvertent stimulation of extrasynaptic/NR2B pro-death NMDARs, we included the NR2BR blocker Ro 25-6981 (6 mg/kg, i.v.) in the treatment regimen. Surprisingly, we found an obvious trend for glycine-mediated neuroprotection even when administered at this delayed time point, though the neuroprotection was not statistically significant due to the small sample size. Nevertheless, this finding supported our hypothesis that stimulation of NMDAR survival signaling pathways may confer a clinically applicable wide therapeutic window, especially in comparison to NMDAR blockers that have failed clinical studies due to their narrow time windows.
3.2.3 Glycine treatment: neuroprotection against global ischemia

We next sought to study the pathological processes of global ischemia, and to examine whether stimulating the NMDAR survival pathway using glycine is also effective against neurological damages resulting from global ischemia. Global ischemia is induced by a prolonged period of cerebral hypo-perfusion. It can be triggered accidentally by sudden cardiac arrest, cardiac deficiency resulting from failure or arrhythmia, or severe blood loss due to traumatic injury. It can also be triggered predictably following cardiac surgeries that required the use of the cardiopulmonary bypass (CPB) pump (a.k.a. “the pump”), such as the coronary-bypass surgery. Clinical features of global ischemia include selective hippocampal degeneration and memory loss. Likewise, experimental induction of global ischemia in rodents triggers bilateral neurodegeneration in the CA1 region of the hippocampus, as well as behavioral deficits in learning and memory tasks (Wood et al., 1992; Wood et al., 1993b). Moreover, like focal ischemic stroke, neurological deficits caused by global ischemia can be rescued by pre-treating the rat with NMDAR antagonists (Wood et al., 1992, 1993a), suggesting that NMDAR-mediated excitotoxicity also contributes to neurological damage following global ischemia.

Since the pathological properties associated with global ischemia has not been well studied in the field, and especially not in the present laboratory, we aimed to first investigate the key features of global ischemia (FIGURES 3-6 and 3-7). Global ischemia was induced by 10 min of transient bilateral carotid arterial occlusion coupled to blood-withdrawal-induced hypotension. We found that reducing blood pressure to 30-35 mmHg results in reproducible global ischemic damage with almost no mortality, in comparison to 20-30 mmHg that results in 30% mortality and to 40-50 mmHg that induces ischemic damage only in 20% of the animals (data not shown). As illustrated with Cresyl Violet staining for viable cells and
condensed cell debris/particles (FIGURE 3-6, A and B), Fluoro Jade-B staining for degenerating neurons (FIGURE 3-6, C-E), and NeuN immunohistochemistry for viable neurons (FIGURE 3-6, F and G), global ischemia induced neuronal death that is specific to CA1 pyramidal neurons of the hippocampus. The rest of the hippocampus and forebrain appeared largely intact. Moreover, the damage appeared to be consistently delayed to 3 days post-ischemia, with little neuronal damage evident in the first 2 days. We next sought to study the effect of global ischemia on glutamatergic synaptic transmission in the Schaffer collateral-to-CA1 pyramidal pathway, where global ischemic damage occurs (FIGURE 3-7). Global ischemia induced a long-term depression (LTD) of excitatory post-synaptic potential (EPSP) in vivo with features similar to classical low-frequency stimulation (LFS) induced LTD, a memory substrate and also a death-signaling mechanism downstream of NR2BRs (see discussion in Chapter 1.5.2). Interestingly, although neuronal death following global ischemia was much delayed, this ischemia-induced LTD (iLTD) occurred soon after global ischemia, and lasted for at least 6 h post-ischemia (data not shown). Whether this iLTD contributed to the actual neuronal death process remains to be tested in future studies with the Tat-GluR2-3Y peptide that selectively blocks LFS-induced LTD (sLTD) (see Chapter 1.5.2 for discussion on Tat-GluR2-3Y peptide and LTD-mediated neuronal death), but will be beyond the scope of this thesis.

In the present study, we then sought to determine whether glycine is also neuroprotective against global ischemia. Rats subjected to 10-min global ischemia were injected with glycine (800 mg/kg, i.p.) or saline control, and euthanized for histological examination 5 days, 7 days, and 21 days post-ischemia (FIGURE 3-8). The dose of glycine used and time of glycine administration were based on the above study for focal ischemic stroke (see Chapter 3.2.3). As expected, glycine was consistently neuroprotective at each of the time points studied.
Given that neuronal death in this animal model continues for up to 21 days post-ischemia (see **FIGURE 3-6** and discussed above), we sought to investigate whether repeated chronic stimulation of the *NMDAR survival pathway* by glycine can be even more neuroprotective (**FIGURE 3-9**). Indeed, repeated daily injections of glycine (800 mg/kg/day x 5 d; i.p.; with the first dose 3 h post-ischemia) resulted in greater neuroprotection (40% protection, see **FIGURE 3-9**) than the single dose regimen (20-25% protection, see **FIGURE 3-8**). In addition, consistent with the notion that glycine act by stimulating the *NMDAR survival pathway*, the effect of glycine was blocked by co-administration of the NMDAR blocker MK-801 (1 mg/kg, i.p.), which had no neuroprotective effect on its own (**FIGURE 3-10**). Finally, in line with the NMDAR dual-role hypothesis, the neuroprotective effect of glycine was significantly reduced by the NR2AR blocker NVP-AAM077 (2.4 mg/kg, i.p.), but not by the NR2BR blocker Ro 25-6981 (6 mg/kg, i.p.) (**FIGURE 3-11**). These experiments suggested that glycine, by stimulating pro-survival NMDARs, is neuroprotective against not only focal ischemic stroke, but also global ischemia.

Because glycine acts by stimulating NMDARs, its neuroprotective effect would only last as long as functional NMDARs remain. We therefore immuno-stained the brains from rats subjected to global ischemia with antibodies against the obligatory NMDAR subunit NR1 (**FIGURE 3-12**). Similar to the timeline of neuronal degeneration following stroke, NR1 remained highly expressed in the CA1 region of the hippocampus for at least 2 days following global ischemia, but abruptly disappeared on day 3 likely in part due to profound neurodegeneration in this area. Although this does not exclude the need for a proper efficacy study to delineate to the therapeutic time window for glycine administration, it provided important mechanistic evidence for why repeated daily doses of glycine was more neuroprotective than the single dose regimen.
TABLE 3-1. Animal Body Temperature before and after surgical induction of middle cerebral arterial occlusion (MCAO). Refer to FIGURES 3-2 and 3-3 for data.

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Glycine</th>
<th>Glycine + MK-801</th>
<th>MK-801</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Weight</td>
<td>323 ± 4 g</td>
<td>322 ± 3 g</td>
<td>325 ± 5 g</td>
<td>326 ± 3 g</td>
</tr>
<tr>
<td>Temperature before MCAO surgery</td>
<td>37.1 ± 0.0 °C</td>
<td>37.1 ± 0.1 °C</td>
<td>37.0 ± 0.1 °C</td>
<td>37.2 ± 0.1 °C</td>
</tr>
<tr>
<td>Temperature after MCAO surgery</td>
<td>36.7 ± 0.1 °C</td>
<td>36.8 ± 0.1 °C</td>
<td>36.7 ± 0.3 °C</td>
<td>36.8 ± 0.1 °C</td>
</tr>
<tr>
<td>Temperature before reperfusion</td>
<td>38.7 ± 0.1 °C</td>
<td>38.5 ± 0.1 °C</td>
<td>38.5 ± 0.2 °C</td>
<td>38.8 ± 0.2 °C</td>
</tr>
<tr>
<td>Temperature after reperfusion</td>
<td>38.2 ± 0.1 °C</td>
<td>38.1 ± 0.2 °C</td>
<td>38.1 ± 0.3 °C</td>
<td>38.3 ± 0.1 °C</td>
</tr>
</tbody>
</table>

TABLE 3-2. Animal Body Temperature before and after surgical induction of middle cerebral arterial occlusion (MCAO). Refer to FIGURE 3-5 for data.

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Glycine + Ro</th>
</tr>
</thead>
<tbody>
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<td>Rat Weight</td>
<td>346 ± 4 g</td>
<td>346 ± 3 g</td>
</tr>
<tr>
<td>Temperature before MCAO surgery</td>
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<td>37.2 ± 0.6 °C</td>
</tr>
<tr>
<td>Temperature after MCAO surgery</td>
<td>36.8 ± 0.1 °C</td>
<td>36.7 ± 0.2 °C</td>
</tr>
<tr>
<td>Temperature before reperfusion</td>
<td>38.3 ± 0.2 °C</td>
<td>38.6 ± 0.0 °C</td>
</tr>
<tr>
<td>Temperature after reperfusion</td>
<td>38.1 ± 0.1 °C</td>
<td>37.9 ± 0.2 °C</td>
</tr>
</tbody>
</table>
FIGURE 3-1. NMDAR subtypes have differential roles in ischemic stroke outcome.
FIGURE 3-1. NMDAR subtypes have differential roles in ischemic stroke outcome.

The two major NMDAR subtypes in the adult forebrain, NR2A/NMDARs (NR2ARs) and NR2B/NMDARs (NR2BRs), had opposing roles in minimizing and exacerbating neuronal damage, respectively, following stroke. Rats were subjected to 1-h middle cerebral arterial occlusion (MCAO) using the intraluminal suture-insertion technique. Their neurological deficits were examined immediately before the rats were euthanized 24 h post-ischemia. Coronal brain (2mm-thick) slice were stained for 10 min with TTC (2,3,5-triphenyltetrazolum). NVP-AAM077 (2.4 mg/kg, i.v.; 30 min before MCAO; n=5) exacerbated, and Ro 25-6981 (6 mg/kg, i.v.; 30 min before MCAO; n=6) protected against cerebral infarction (A and B) and behavioral deficits (C), suggesting opposing and respective roles of NR2ARs and NR2BRs in neuronal survival and death during stroke.

Data is expressed as mean ± sem.

Statistical Analysis:
One Way ANOVA detected significant difference between the treatment groups (P<0.001), and post-hoc comparisons by the Holm Sidak method revealed significant increase in infarct volume in the NVP-treated group (*P<0.05) and significant decrease in the Ro-treated group (***P<0.01).

(*The work presented in this figure was done by Drs Yitao Liu and Dong Chuan Wu. Source: Liu et al. J Neurosci 2007)
FIGURE 3-2. Glycine protected the rat from sensory and motor deficits following focal ischemic stroke.
FIGURE 3-2. Glycine protected the rat from sensory and motor deficits following focal ischemic stroke.

C

D
FIGURE 3-2. Glycine protected the rat from sensory and motor deficits following focal ischemic stroke.
FIGURE 3-2. Glycine protected the rat from sensory and motor deficits following focal ischemic stroke.

Selective stimulation of pro-survival NMDARs, those in the synapse and contain the NR2A subunit, was achieved by administration of the NMDAR co-agonist glycine at a time point when glutamate would only be present in the synapses. Rats were subjected to 90-min middle cerebral arterial occlusion (MCAO) using the intraluminal suture-insertion technique. Their neurological deficits were examined during the ischemic period (+1 h post-ischemia onset), prior to glycine treatment 2.5 h post-reperfusion (+4 h post-ischemia onset), and immediately before the rats were euthanized +24 h post-ischemia onset. Glycine (800 mg/kg, i.p.; 4.5 h after MCAO onset; n=8) protected the rat against some motor and sensory deficits, compared to control animals that received saline (i.p.; n=8). The left/right motor symmetry test (A) and the visual side test (B) examined motor capacity, and the proprioceptive test (C) and the tactile side test (D) examined sensory capacity. In addition, the visual forward test (E) examined motor preference, and the tactile forward test (F) examined sensory preference. Data is expressed as mean ± sem.
FIGURE 3-3. Glycine protected the rat brain from cerebral infarction following focal ischemic stroke.

A

B

C

In the timeline (A), the sequence of events is as follows:
- Occlusion (Ischemia) at 1.5 hours
- Reperfusion +1.5 hrs
- Intraperitoneal Injection +4.5 hrs
- Sacrifice Animal +24 hrs

The graph (B) shows the infarct area (mm$^2$) as a function of distance from Bregma (mm), with different treatments: Saline, Glycine, Glycine + MK801, and MK801. The y-axis represents the infarct area, and the x-axis represents the distance from Bregma.

The bar graph (C) compares the infarct volume (% relative to control) across different treatments: Saline, Glycine + MK801, and MK801. The bar marked with an asterisk (*) indicates a significant difference from the control group.
FIGURE 3-3. Glycine protected the rat brain from cerebral infarction following focal ischemic stroke.

D

Saline

Glycine

Glycine + MK801

MK-801
**FIGURE 3-3.** Glycine protected the rat brain from cerebral infarction following focal ischemic stroke.

Selective stimulation of the pro-survival (synaptic/NR2A) NMDARs was achieved by administration of the NMDAR co-agonist glycine at a time point when glutamate would only be present in the synapses. Rats were subjected to 90-min middle cerebral arterial occlusion (MCAO) using the intraluminal suture-insertion technique. Coronal brain (2mm-thick) slice were collected 24 h post-ischemia, and stained for 10 min with TTC (2,3,5-triphenyltetrazolum). Glycine (800 mg/kg, i.p.; 4.5 h post-MCAO onset; n=8) protected the rat against cerebral infarction, compared to control animals that received saline treatment (i.p.; n=8). This neuroprotective effect of glycine was blocked by simultaneous co-administration of MK-801 (1 mg/kg; i.p.; n=7), which on its own had little effect on stroke outcome (n=8). A, timeline. B and C, summarized data. D, representative images for TTC staining. Data is expressed as mean ± sem.

Statistical Analysis:

**B,** Two Way Repeated Measure ANOVA (with “infarct-area” repetition) detected significant effect of treatment (F=4.291 and p=0.013) and significant differences in infarct area across the brain (F=167.339 and p<0.001). There was no significant effect of treatment on the pattern of infarct area across the brain (F=1.269 and p=0.230). Post-hoc pairwise multiple comparisons by the Holm-Sidak method confirmed significant neuroprotection by glycine (*P=0.010).

**C,** One Way ANOVA detected significant effect of treatment (F=4.179 and P=0.015), and post-hoc pairwise comparisons by the Holm-Sidak method detected significant neuroprotection by glycine (*P=0.007).
FIGURE 3-4. Neuroprotection against focal ischemia by glycine required NR2ARs but not NR2BRs.

A

1.5 hours  3 hours  22.5 hours
Occlusion (Ischemia)  Reperfusion  Intraperitoneal Injection
+1.5 hrs  +4.5 hrs
Sacrifice Animal +24 hrs

B

Infarct Volume (% relative to control)

Control  Ro  Gly+Ro  Gly+Ro+NVP

**

C

Control  Ro  Gly+Ro  Gly+Ro+NVP
**FIGURE 3-4. Neuroprotection against focal ischemia by glycine required NR2ARs but not NR2BRs.**

The two major NMDAR subtypes in the adult forebrain, NR2A/NMDARs (NR2ARs) and NR2B/NMDARs (NR2BRs), had opposing roles in minimizing and exacerbating neuronal damage, respectively, following stroke. Rats were subjected to 90-min middle cerebral arterial occlusion (MCAO) using the intraluminal suture-insertion technique. Coronal brain (2mm-thick) slice were collected 24 h post-ischemia, and stained for 10 min with TTC (2,3,5-triphenyltetrazolum). Glycine (800 mg/kg, i.p.; 4.5 h post-MCAO onset; n=9) with Ro 25-6981 (6 mg/kg, i.p.) protected the rat against cerebral infarction, compared to control animals that received saline treatment (i.p.; n=10) or Ro 25-6981 alone (n=10). This neuroprotective effect of glycine was blocked by co-administration of NVP-AAM077 (2.4 mg/kg; i.p.; n=10).  


Data is expressed as mean ± sem.  

Statistical Analysis:  

One Way ANOVA detected significant difference between treatment groups (P<0.001), and post-hoc multiple comparisons by the Holm-Sidak method found significant neuroprotection by Glycine + Ro (*P<0.001)  

(*The work presented in this figure was done by Drs Yitao Liu and Dong Chuan Wu. Source: Liu et al. J Neurosci 2007)
FIGURE 3-5. Glycine remained neuroprotective against cerebral infarction when administered up to 6 h post-focal ischemic stroke.
FIGURE 3-5. Glycine remained neuroprotective against cerebral infarction when administered up to 6 h post-focal ischemic stroke.

Selective stimulation of pro-survival NMDARs, those in the synapse and contain the NR2A subunit, was achieved by administration of the NMDAR co-agonist glycine at a time point when glutamate would only be present in the synapses. Rats were subjected to 90-min middle cerebral arterial occlusion (MCAO) using the intraluminal suture-insertion technique. Coronal brain (2mm-thick) slice were collected 24 h post-ischemia, and stained for 10 min with TTC (2,3,5-triphenyltetrazolum). Glycine (800 mg/kg) + Ro 25-6981 (6mg/kg, i.p.; 6 h post-MCAO onset; n=4) protected the rat against cerebral infarction, compared to control animals that received saline treatment (i.p.; n=3).


Data is expressed as mean ± sem.

Statistical Analysis:

B, Two Way Repeated Measure ANOVA (with “infarct-area” repetition) detected no significant effect of treatment (F=5.466 and P=0.067) and significant differences in infarct area across the brain (F=26.893 and P<0.001). There was no significant effect of treatment on the pattern of infarct area across the brain (F=2.100 and P=0.099).

C, t test detected no significant effect of the treatment (t=2.338 and P=0.067) compared to the control group.
FIGURE 3-6. Global ischemia induced delayed neuronal death in the CA1 region of the hippocampus.
FIGURE 3-6. Global ischemia induced delayed neuronal death in the CA1 region of the hippocampus.
FIGURE 3-6. Global ischemia induced delayed neuronal death in the CA1 region of the hippocampus.

B

![Graph showing number of viable cells, number of cell debris particles, and number of surrounding debris particles over time post-global ischemia for CA1 and CA3 regions.](image)
FIGURE 3-6. Global ischemia induced delayed neuronal death in the CA1 region of the hippocampus.

C

D

E

FIGURE 3-6. Global ischemia induced delayed neuronal death in the CA1 region of the hippocampus.

C

D

E
FIGURE 3-6. Global ischemia induced delayed neuronal death in the CA1 region of the hippocampus.
FIGURE 3-6. Global ischemia induced delayed neuronal death in the CA1 region of the hippocampus.

G

![Graph showing the number of NeuN+ cells over time post-global ischemia for CA1 and CA3 regions.](image)

- **CA1**
- **CA3**

Number of NeuN+ cells

Time post-global ischemia

0 1 2 3 4 5 6 7 14 21
**FIGURE 3-6. Global ischemia induced delayed neuronal death in the CA1 region of the hippocampus.**

Global ischemia induced neuronal death that was selective to the CA1 region of the hippocampus, and was delayed for 3 days post-ischemia. Rats were subjected to 10-min global ischemia induced by bilateral carotid arterial occlusion coupled to blood-withdrawal-induced hypotension, and euthanized and fixed by perfusion with paraformaldehyde at different time points to allow histological examination of neuronal death in the hippocampus. Coronal brain slices were stained with Cresyl Violet (A and B) for nuclei of viable (rounded) and degenerating cells (condensed and irregular shaped), Fluoro-Jade B (C, D, and E) for neurons undergoing neurodegeneration, or NeuN for mature neurons (F and G).

A and B, Representative images (A) and summarized data (B) for Cresyl Violet staining. C, D, and E, Representative images (C) and summarized data (D and E) for Fluoro-Jade B staining. F and G, Representative images (F) and summarized data (G) for NeuN immunohistochemistry.

Data is expressed as mean ± sem. Scale bar = 50μm or 1000μm.

**Statistical Analysis:**

**B top panel,** Two Way ANOVA detected significant effect of global ischemia on the number of viable cells over time (F=27.845 and P<0.001) and significant differences in the number of viable cells between different brain regions (F=190.887 and P<0.001). Global ischemia also produces significantly different effect at different brain regions (F=28.709 and P<0.001). Post-hoc pairwise comparisons by the Holm-Sidak method detected significant neurodegeneration in the CA1, but not in the CA3 region, region starting 2 days post-ischemia (P<0.05).
D. One Way ANOVA detected significant time-dependent effect of global ischemia on the area of neurodegeneration stained with Fluoro Jade B (F=18.236 and P<0.001) and significant differences in the number of viable cells between different brain regions (F=190.887 and P<0.001). Post-hoc pairwise comparisons by the Holm-Sidak method detected significant expansion of the Fluro-Jade-positive area starting 3 days post-ischemia (P<0.010).

E. One Way ANOVA detected significant time-dependent effect of global ischemia on the intensity of Fluoro Jade staining (F=8.449 and P<0.001). Post-hoc pairwise comparisons by the Holm-Sidak method detected significant increase in the intensity starting 5 days post-ischemia (P<0.010).

G. Two Way ANOVA detected significant effect of global ischemia on the number of NeuN+ cells over time (F=5.579 and P<0.001), and no significant difference in the number of NeuN+ cells between different brain regions (F=3.863 and P=0.053).
FIGURE 3-7. Global ischemia induced long term depression (i-LTD) of synaptic efficacy in the Schaffer collateral-to-CA1 pathway.
FIGURE 3-7. Global ischemia induced long term depression (i-LTD) of synaptic efficacy in the Schaffer collateral-to-CA1 pathway.

Global ischemia induced long term depression (i-LTD) with features similar to long-frequency stimulation (LFS) induced LTD (s-LTD). Rats under pentobarbital anesthesia were subjected recording of excitatory post-synaptic potential (EPSP) in the Schaffer collateral-to-CA1 pathway. Following stabilization of EPSP baseline, each rat was subjected to 10-min global ischemia by bilateral carotid arterial occlusion coupled to blood withdrawal-induced hypotension. Global ischemia induced complete loss of synaptic activity during the 10-min ischemic period, which was quickly recovered following reperfusion. Despite re-oxygenation, the EPSP remained partially depressed, and this i-LTD lasted for at least 2 h post-ischemia. In selected animals, prolonged monitoring of EPSP showed that this i-LTD could last up to 6 h post-ischemia (data not shown).

Data is expressed as mean ± sem.

Statistical Analysis:

Two Way Repeated Measure ANOVA (with “time” repetition) detected significant effect of global ischemia on changes in EPSP over time (F=7.741 and P<0.001). Overall, there were significant changes in EPSP over time (F=9.142 and P<0.001) and significant effect of global ischemia on EPSP (F=42.979 and P<0.001). Post-hoc comparisons using the Holm-Sidak method found significant depression of EPSP following global ischemia (P<0.050) but number following sham operation.
FIGURE 3-8. Glycine produced long lasting protection against global ischemia-induced CA1 neurodegeneration.
FIGURE 3-8. Glycine produced long lasting protection against global ischemia-induced CA1 neurodegeneration.

C

![Graph showing the number of Fluoro-Jade positive neurons at different time points after ischemia.]

- 5 days post-ischemia
- 7 days post-ischemia
- 21 days post-ischemia

D

![Graph showing the percentage of Fluoro-Jade positive neurons relative to saline-control at different time points after ischemia.]

- 5 days post-ischemia
- 7 days post-ischemia
- 21 days post-ischemia

* indicates statistical significance compared to saline-control.
FIGURE 3-8. Glycine produced long lasting protection against global ischemia-induced CA1 neurodegeneration.

Selective stimulation of pro-survival NMDARs, those in the synapse and contain the NR2A subunit, was achieved by administration of the NMDAR co-agonist glycine at a time point when glutamate would only be present in the synapses. Rats were subjected to 10-min global ischemia induced by bilateral carotid arterial occlusion coupled to blood-withdrawal-induced hypotension, and they were euthanized and fixed by perfusion with paraformaldehyde at different time points to allow histological examination of neuronal death in the hippocampus. Coronal brain slices were stained with Fluoro-Jade B for neurons undergoing neurodegeneration. Glycine (800 mg/kg, i.p.; 3 h post-ischemia) protected the rat against cerebral infarction, compared to control animals that received saline treatment, at all time points examined.

A, timeline. B, representative images for Fluoro-Jade B staining. C and D, summarized data expressed as the absolute number of Fluoro Jade-positive neurons (C) and the percentage ratio compared to control (D).

Data is expressed as mean ± sem. Scale bar = 100µm or 1000µm.

Statistical Analysis:

C, Two Way ANOVA detected significant effect of glycine on the number of degenerating neurons in rats subjected to global ischemia (F=16.407 and P<0.001), and significant difference in the number of degenerating cells at different time points post-ischemia (F=12.069 and P<0.001). In addition, significant effect of the treatment on region differences was not detected (F=0.188 and P=0.830). Post-hoc pairwise comparisons by the Holm-Sidak method detected significant neuroprotection by glycine overall (t=4.051 and P<0.001), when
the animal is sacrificed 5 days (t=2.167 and P=0.037) or 7 days (t=3.283 and P=0.002), but not 21 (=1.832 and P=0.075) days after global ischemia.

D. Two Way ANOVA detected significant effect of glycine on the number of degenerating neurons in rats subjected to global ischemia (F=16.972 and P<0.001), and this was regardless of the time of sacrifice (F=0.182 and P=0.834). Post-hoc pairwise comparisons by the Holm-Sidak method detected significant neuroprotection by glycine when the animal is sacrificed 5 days or 7 days, but not 21 days after global ischemia (*P<0.05).
FIGURE 3-9. Repeated administration of glycine resulted in augmented neuroprotection against global ischemia.
FIGURE 3-9. Repeated administration of glycine resulted in augmented neuroprotection against global ischemia.

When given repeatedly, glycine produced augmented neuroprotection against global ischemia. Rats were subjected to 10-min global ischemia induced by bilateral carotid arterial occlusion coupled to blood-withdrawal-induced hypotension, and they were euthanized and fixed by perfusion with paraformaldehyde at 5 days post-ischemia to allow histological examination of neuronal death in the hippocampus. Coronal brain slices were stained with Fluoro-Jade B for neurons undergoing neurodegeneration. Glycine (800 mg/kg x 5, i.p.; first dose at 3 h post-ischemia) substantially protected the rat against cerebral infarction, compared to control animals that received saline treatment, at all time points examined.

A, timeline. B, summarized data showing number of Fluoro-Jade B positive neurons. Data is expressed as mean ± sem.

Statistical Analysis:

B, t test detected significant decrease in the number of degenerating neurons (detected by Fluoro Jade B) by repeated doses of glycine (t=3.341 and **P=0.009).
FIGURE 3-10. Neuroprotective effect of glycine against global ischemia was NMDAR-dependent.

A

B

<table>
<thead>
<tr>
<th>NeuN</th>
<th>Saline</th>
<th>Glycine</th>
<th>Glycine + MK-801</th>
<th>MK-801</th>
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</thead>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>FluoroJade</th>
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<th>Glycine + MK-801</th>
<th>MK-801</th>
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<tr>
<td>CA1</td>
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<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>
FIGURE 3-10. Neuroprotective effect of glycine against global ischemia was NMDAR-dependent.

C

![Graph showing Neuroprotective effect of glycine against global ischemia](image)

D

![Graph showing % of FluoroJade positive Neurons](image)
FIGURE 3-10. Neuroprotective effect of glycine against global ischemia was NMDAR-dependent.

Selective stimulation of pro-survival NMDARs, those in the synapse and contain the NR2A subunit, was achieved by administration of the NMDAR co-agonist glycine at a time point when glutamate would only be present in the synapses. Rats were subjected to 10-min global ischemia induced by bilateral carotid arterial occlusion coupled to blood-withdrawal-induced hypotension, and they were euthanized and fixed by perfusion with paraformaldehyde 7 days post-ischemia to allow histological examination of neuronal death in the hippocampus. Coronal brain slices were stained with Cresyl Violet for nuclei of viable (rounded) and degenerating cells (condensed and irregular shaped), Fluoro-Jade B for neurons undergoing neurodegeneration, or immuno-stained for NeuN for viable neuronal nuclei. Glycine (800 mg/kg, i.p.; 3 h post-ischemia; n=9) protected the rat against cerebral infarction, compared to control animals that received saline treatment (n=10). This neuroprotective effect of glycine was blocked by simultaneous co-administration of MK-801 (1 mg/kg; i.p.; n=10), which on its own had little effect on stroke outcome (n=11).

A, timeline. B, C, and D, representative images (B) and summarized data (C and D) for NeuN immunohistochemistry and Fluoro Jade B staining.

Data is expressed as mean ± sem. Scale bar = 100μm or 1000μm.

Statistical Analysis:

C, One Way ANOVA detected no significant effect of treatment on the number of NeuN-positive cells (F=1.131 and *P=0.3617).

D, One Way ANOVA detected significant effect of treatment on the number of Fluoro Jade-positive neurons (F=7.310 and *P<0.001). Post-hoc multiple comparison procedures using the
Holm-Sidak method found significant neuroprotection by glycine compared to saline (t=3.660 and *P=0.004), and also significant inhibition of glycine’s effect by MK801 (glycine + MK801) compared to glycine alone (t=4.325 and P<0.001).
FIGURE 3-11. Neuroprotection against global ischemia by glycine required NR2ARs but not NR2BRs.
FIGURE 3-11. Neuroprotection against global ischemia by glycine required NR2ARs but not NR2BRs.

The two major NMDAR subtypes in the adult forebrain, NR2A/NMDARs (NR2ARs) and NR2B/NMDARs (NR2BRs), had opposing roles in minimizing and exacerbating neuronal damage, respectively, following stroke. Rats were subjected to 10-min global ischemia induced by bilateral carotid arterial occlusion coupled to blood-withdrawal-induced hypotension, and they were euthanized and fixed by perfusion with paraformaldehyde 7 days post-ischemia to allow histological examination of neuronal death in the hippocampus. Coronal brain slices were stained with Fluoro-Jade B for neurons undergoing neurodegeneration. Glycine (800 mg/kg, i.p.; 3 h post-ischemia) alone (n=5) or in combination with Ro 25-6981 (6 mg/kg, i.p.; n=5) protected the rat against cerebral infarction, compared to control animals that received saline treatment (n=4). This neuroprotective effect of glycine was blocked by simultaneous co-administration of NVP-AAM077 (2.4 mg/kg; i.p.; Gly+NVP, n=6; Gly+Ro+NVP, n=5), which on its own has little effect on stroke outcome (n=4).

Statistical Analysis:

B, One Way ANOVA detected significant effect of treatment on the number of Fluoro Jade-positive neurons (F=4.690 and *P=0.003). Post-hoc multiple comparison procedures using the Holm-Sidak method found significant neuroprotection by glycine (t=3.657 and *P=0.007) and glycine + Ro 25-6981 (t=3.608 and *P=0.007).
FIGURE 3-12. NR1 protein expression in the CA1 region following global ischemia.

Control CA1

1 Day post-ischemia
FIGURE 3-12. NR1 protein expression in the CA1 region following global ischemia.

2 Days post-ischemia 3 Days post-ischemia

NR1

DAPI

NR1

DAPI
FIGURE 3-12. NR1 protein expression in the CA1 region following global ischemia.

The obligatory NMDAR subunit NR1 remained highly expressed in the CA1 region of the rat hippocampus for up to 2 days post-global ischemia. Rats were subjected to 10-min global ischemia induced by bilateral carotid arterial occlusion coupled to blood-withdrawal-induced hypotension, and they were euthanized and fixed by perfusion with paraformaldehyde at different time points to allow histological examination of neuronal death in the hippocampus. Coronal brain slices were immuno-stained for NR1.

Scale Bar = 100μm.
CHAPTER 4:
INHIBITING NR2B-TO-SREBP1 SIGNALING

4.1 Specific Background

4.1.1 Excitotoxic role of SREBP1

Lipid metabolism in the brain has critical roles in supporting neuronal survival and dysfunction of lipid metabolism may contribute to neuronal injuries following brain insults such as stroke. As discussed in Chapter 1.5.4, SREBP1 is a membrane-bound transcription factor involved in the regulation of lipid synthesis/metabolism both in peripheral tissues and in the brain. Yet little is known about its dysfunction in mediating neuronal injuries following brain insults such as a stroke. We have recently reported the first evidence that SREBP1 contributes to excitotoxic neuronal death mediated by NR2BR stimulation (Lai T.W., 2009, 2007; Taghibiglou et al., 2009). By means of screening transcription factors against their characterized transcriptional binding sites, we identified a significant increase in SREBP1 transcriptional binding activity following excitotoxic neuronal insult. Post hoc western blot and immunofluorescence analysis further confirmed SREBP1 activation and translocation to the nucleus following excitotoxic NMDAR stimulation in cultured cortical neurons, and that this activation of SREBP1 is secondary to the ubiquitination and proteasomal degradation of its inhibitory binding partner INSIG1 (protein encoded by the insulin signaling gene-1). Consistent with a critical role of SREBP1 in mediating neuronal injuries following the ischemic challenge, we found that preventing SREBP1 activation protected neurons against excitotoxic neuronal death.
4.1.2 The development of INDIP

Since it would be therapeutically impractical to knockdown SREBP1 with siRNA or to inhibit SREBP1 by mass supplying cholesterol, we sought to develop a peptide that act by inhibiting SREBP1 activation. As described earlier, excitotoxic activation of SREBP1 requires ubiquitination and degradation of its inhibitory binding partner insig1 (see Chapter 4.1.1). Because this insig1-to-SREBP1 signaling cascade and the specific ubiquitination sites for insig1 have already been reported, we are able to design an interference peptide, named INDIP (insig1 degradation inhibiting peptide, with sequence: GEPHKFKREW or INSIG1 152-161), that selectively inhibits SREBP1 activation by preventing insig1 degradation. The peptide has the same amino-acid sequence as the 10 amino-acid residues flanking the insig1 ubiquitination sites K156 and K158. To make INDIP membrane-permeable and BBB-permeable for in vivo administration, we further included a MTD (sequence: YGRKKRRQRRR) of the HIV1 Tat protein (see Chapter 1.6.3 for a brief introduction to Tat-linked peptides). As control, we also designed a scrambled peptide Tat-INDIP-Mix and an ubiquitination-site mutant peptide Tat-INDIP-K>R.

As expected, Tat-INDIP but not the control peptides prevents NMDAR-mediated insig1 ubiquitination and degradation in cultured cortical neurons (Taghibiglou et al., 2009), and thereby inhibits SREBP1 activation. Moreover, the effect of Tat-INDIP on inhibition of insig1 ubiquitination seems to be rather selective, as it does not affect ubiquitination of other proteins (Taghibiglou et al., 2009).
4.2 Results

4.2.1 SREBP1 activation following stroke

Although NMDAR blockers are effective against ischemic neuronal death, they are not clinically feasible in large due to their prominent side effects and their narrow therapeutic window (see Chapter 1.6.2 for thorough discussion). As discussed in Chapters 1.4-1.6, stroke research in recent years has focused on developing therapeutic peptides that selectively inhibit death-signaling proteins downstream of NMDARs. Our laboratory recently identified SREBP1 as one such protein, whose activation may be delayed for up to 6 h after NMDAR stimulation (Lai T.W., 2009, 2007; Taghibiglou et al., 2009). Consistent with the neuronal death role of NR2BRs, excitotoxic activation of SREBP1 requires NR2BRs and not NR2ARs (Taghibiglou et al., 2009). Finally, in vitro evidence based on SREBP1 inhibition with cholesterol and SREBP1 knockdown by siRNA strongly suggests that SREBP1 is critically important for NMDAR-mediated neuronal death (Taghibiglou et al., 2009). To investigate whether SREBP1 can also account for neuronal injuries following focal ischemic stroke, rats subjected to MCAO were euthanized 24 h post-ischemia to allow western blot analysis of SREBP1 in both whole cell lysate and nuclear fraction (FIGURE 4-1). Indeed, MCAO in rats induced substantial activation of SREBP1, as denoted by the shorter active N terminus fragment (nt-SREBP1), specifically in the ischemic hemisphere but not the contralateral hemisphere. Consistent with increased SREBP1 transcriptional activity, nt-SREBP1 was substantially increased in the nuclear fraction of the ischemic hemisphere following MCAO. These findings, together with previous findings (Taghibiglou et al., 2009), suggested that excitotoxic NMDAR stimulation during stroke activates SREBP1, which becomes cleaved into the active nt-SREBP1 fragment that translocates to the cell nucleus to promote neuronal death.
4.2.2 Inhibiting SREBP1 activation by Tat-INDIP and neuroprotection

Since it is clinically impractical to knockdown SREBP1 by siRNA or inhibiting SREBP1 by oversupply cholesterol, we developed a therapeutic peptide named INDIP (see Chapter 4.1.2) (FIGURE 4-2). To investigate whether this peptide indeed can prevent insig1 degradation and thereby SREBP1 activation following stroke, the Tat-linked version of INDIP (Tat-INDIP) was administered to rats 45 min before subjecting them to MCAO (FIGURE 4-3). The rats were euthanized 3 h after MCAO to allow tissue collection for western blot analysis of insig1. Here, Tat-INDIP (8 mg/kg, i.v.) but not the control peptide Tat-INDIP-K>R (8 mg/kg) completely abolished MCAO-induced insig1 degradation (Figure 4-3, B and C). Since this 3-h time point was too early for determining whether inhibition of insig1 by Tat-INDIP can be translated to an inhibition of SREBP1 activation, we repeated this experiment except this time the rats were euthanized 24-h post-MCAO – a time point at which we previously found profound SREBP1 activation. Indeed, the inhibition of insig1 degradation by Tat-INDIP (8 mg/kg, i.v.) was translated into a profound inhibition of SREBP1 activation (compared to control, MCAO only) (FIGURE 4-3, D and E).

To investigate whether SREBP1 inhibition by Tat-INDIP is indeed beneficial to the ischemic brain, rats subjected to 90-min MCAO were injected with Tat-INDIP at the same dose and regimen previously shown to be effective at preventing insig1 degradation and SREBP1 activation, and they were subjected to neurological examination (FIGURE 4-4) and euthanized 24 h post-ischemia to allow histological examination of cerebral infarction (FIGURE 4-5). Even though Tat-INDIP (8 mg/kg, i.v.; 45 min prior to MCAO) was administered prior to MCAO, it had no effect on neurological performance during the occlusion period (FIGURE 4-4). This was expected, since neurological deficits during the
occlusion period should reflect neuronal inactivity due to the vascular occlusion, rather than neuronal death. In marked contrast, it significantly improved neurological outcome when examined 24 h post-ischemia, when control animals that received saline remained severely impaired. Most impressively, Tat-INDIP (8 mg/kg, i.v.; 45min prior to MCAO) profoundly decreased cerebral infarction by \(~50\%\), compared to control animals treated with saline (i.v.) or the scrambled peptide Tat-INDIP-Mix (8 mg/kg, i.v.) (FIGURE 4-5). This result strongly suggested that SREBP1 is critical for excitotoxic neuronal death following stroke, and that its inhibition by Tat-INDIP is a novel and innovative NMDAR-based therapeutic strategy for stroke intervention.

4.2.3 Preclinical evaluation of Tat-INDIP for post-stroke therapy

The failure of NMDAR blockers in clinical studies has been largely attributed to the narrow therapeutic window associated with these compounds. As discussed in Chapter 1.6.2, once the death signaling proteins downstream of NMDARs are activated, blocking these receptors is no longer useful. Indeed, NMDAR blockers have been reported to lose efficacy when the treatment is delayed for more 30 min to 1 h post-ischemia in animal models of stroke (Gerasimov et al., 2004; Liu et al., 2007; Schulz et al., 1995). Based on the understanding SREBP1 activation is far downstream of NR2BR activation (Taghibiglou et al., 2009), we sought to test whether SREBP1 is effective against stroke when the treatment is delayed for 2 h post-ischemia. Moreover, because some experimental compounds are thought to delay rather than prevent neuronal death, we aimed to test whether the neuroprotection mediated by Tat-INDIP will remain evident one week after MCAO. Here, Tat-INDIP (8 mg/kg, i.v.) improved neurological outcome over the one-week period, compared to saline control (i.v.), even when the treatment was given 2 h post-MCAO (FIGURE 4-6). By means of
immunofluorescence, we made the surprising discovery that SREBP1 remains strongly activated one week following MCAO, suggesting room for delayed or repeated therapeutic intervention (FIGURE 4-7). Interestingly, in rats treated with Tat-INDIP, no SREBP1 activation can be seen one week after MCAO/treatment. It is unclear at this point whether this later observation is due solely to neuronal recovery, or partly due to preservation of some peptides in the rat brain.

To analyze the effect of Tat-INDIP on delayed neuronal death one week following MCAO, we stained each coronal brain slice with Fluoro-Jade B that specifically labels actively degenerating neurons (FIGURE 4-8). Consistent with earlier reports that neuronal death following MCAO can be progressive and long lasting, we found substantial Fluoro-Jade Staining in these brain slices. As expected from the strong inhibition of SREBP1, Tat-INDIP (8 mg/kg, i.v.; 2 h post-MCAO onset) but not the mutant peptide Tat-INDIP-K>R (8 mg/kg, i.v.) strongly protected the rat brain against MCAO-induced delayed neuronal death, compared to control animals treated with saline (i.v.). Again, the neuroprotection by Tat-INDIP was very substantial, with an approximately 50% decrease in the size of the infarct volume outlined by Fluoro Jade B staining. These results altogether suggested that Tat-INDIP, with its better time window compared to conventional NMDAR blockers, could be comparatively more clinically feasible for stroke intervention. Moreover, because SREBP1 activation lasted for up to a week following stroke, the therapeutic time window for Tat-INDIP may be substantially wider than the 2-h time point tested in this study.
TABLE 4-1. Average animal body temperature before and after surgical induction of middle cerebral arterial occlusion (MCAO). Refer to FIGURES 4-3, 4-4, and 4-5 for data.

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Tat-INDIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Weight</td>
<td>348 ± 5 g</td>
<td>354 ± 6 g</td>
</tr>
<tr>
<td>Temperature before</td>
<td>36.6 ± 0.2 °C</td>
<td>35.7 ± 0.1 °C</td>
</tr>
<tr>
<td>MCAO surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature after</td>
<td>37.0 ± 0.2 °C</td>
<td>36.5 ± 0.1 °C</td>
</tr>
<tr>
<td>MCAO surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature before</td>
<td>38.6 ± 0.1 °C</td>
<td>38.6 ± 0.1 °C</td>
</tr>
<tr>
<td>reperfusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature after</td>
<td>38.0 ± 0.1 °C</td>
<td>38.3 ± 0.1 °C</td>
</tr>
<tr>
<td>reperfusion</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 4-2. Total combined number of rats used and overall surgery success rate, for the experiments described in FIGURES 4-3, 4-4, and 4-5.

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Tat-INDIP</th>
<th>Tat-INDIP-Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Rats Used</td>
<td>17</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>No Stroke</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>-2</td>
<td>0</td>
<td>-2</td>
</tr>
<tr>
<td>Sample size (n)</td>
<td>15</td>
<td>15</td>
<td>12</td>
</tr>
</tbody>
</table>
TABLE 4-3. Animal body temperature before and after surgical induction of middle cerebral arterial occlusion (MCAO). Refer to FIGURES 4-6, 4-7, and 4-8 for data.

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Tat-INDIP</th>
<th>Tat-Mutated INDIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Weight</td>
<td>356 ± 8 g</td>
<td>357 ± 9 g</td>
<td>359 ± 16 g</td>
</tr>
<tr>
<td>Temperature before MCAO</td>
<td>36.3 ± 0.3 °C</td>
<td>36.4 ± 0.2 °C</td>
<td>26.1 ± 0.3 °C</td>
</tr>
<tr>
<td>Temperature after MCAO</td>
<td>36.7 ± 0.2 °C</td>
<td>36.2 ± 0.4 °C</td>
<td>36.4 ± 0.3 °C</td>
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<tr>
<td>Temperature before reperfusion</td>
<td>37.8 ± 0.1 °C</td>
<td>37.7 ± 0.1 °C</td>
<td>37.7 ± 0.1 °C</td>
</tr>
<tr>
<td>Temperature after reperfusion</td>
<td>37.5 ± 0.1 °C</td>
<td>37.4 ± 0.1 °C</td>
<td>37.3 ± 0.1 °C</td>
</tr>
<tr>
<td>Heart Rate before MCAO</td>
<td>351 ± 16 bps</td>
<td>375 ± 15 bps</td>
<td>366 ± 8 bps</td>
</tr>
<tr>
<td>Heart Rate after MCAO</td>
<td>382 ± 20 bps</td>
<td>371 ± 15 bps</td>
<td>374 ± 10 bps</td>
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<tr>
<td>Heart Rate before reperfusion</td>
<td>413 ± 12 bps</td>
<td>392 ± 10 bps</td>
<td>418 ± 24 bps</td>
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<tr>
<td>Heart Rate after reperfusion</td>
<td>377 ± 10 bps</td>
<td>374 ± 12 bps</td>
<td>368 ± 14 bps</td>
</tr>
<tr>
<td>pO2 before MCAO</td>
<td>96 ± 1 %</td>
<td>96 ± 1 %</td>
<td>96 ± 2 %</td>
</tr>
<tr>
<td>pO2 after MCAO</td>
<td>93 ± 1 %</td>
<td>93 ± 1 %</td>
<td>94 ± 2 %</td>
</tr>
<tr>
<td>pO2 before MCAO</td>
<td>92 ± 1 %</td>
<td>90 ± 1 %</td>
<td>93 ± 1 %</td>
</tr>
<tr>
<td>pO2 before MCAO</td>
<td>92 ± 2 %</td>
<td>87 ± 3 %</td>
<td>92 ± 2 %</td>
</tr>
</tbody>
</table>
TABLE 4-4. Total combined number of rats used and overall surgery success rate, for the experiments described in FIGURES 4-6, 4-7, and 4-8.

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Tat-INDIP</th>
<th>Tat-Mutated INDIP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Rats Used</strong></td>
<td>9</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td><strong>No Stroke</strong></td>
<td>-1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Hemorrhage</strong></td>
<td>0</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td><strong>Sample size (n)</strong></td>
<td>8</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>
FIGURE 4-1. Focal ischemic stroke induces regional SREBP1 activation and nuclear translocation in the brain.

B

<table>
<thead>
<tr>
<th></th>
<th>Sham Rat</th>
<th>Stroke Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cont</td>
<td>Occl</td>
</tr>
<tr>
<td>nt-SREBP1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GluR1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 4-1. Focal ischemic stroke induces regional SREBP1 activation and nuclear translocation in the brain.
FIGURE 4-1. Focal ischemic stroke induces regional SREBP1 activation and nuclear translocation in the brain.

SREBP1 was recently found to be a critical death signaling protein downstream of NR2BRs. To examine whether SREBP1 is indeed activated following stroke, rats were subjected to 90-min middle cerebral arterial occlusion (MCAO) using the intraluminal suture-insertion technique. One day post-ischemia, each rat was euthanized to allow collection of brain tissue from the motor and sensory cortex for western blot analysis (A). Following tissue collection, each brain slice was stained with TTC to ensure that the tissues were collected from the appropriate occluded area (Occl), versus the contralateral control area (Cont). Sham rats received the same surgery, except the ‘MCAO occluder’ did not reach the MCA. The tissues were either prepared as whole-cell lysate (B) or nuclear extract (C). The result showed an increase in the active N-terminus fragment (nt-SREBP1) in both the lysate and nuclear extract, suggesting activation and nuclear translocation of SREBP1.
FIGURE 4-2. Development of the INDIP peptide.
FIGURE 4-2. Development of the INDIP peptide.

<table>
<thead>
<tr>
<th>Tat-INDIP</th>
<th>Tat 47-57</th>
<th>INSIG 152-161</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>(Inhibiting peptide)</em></td>
<td>YGRKKRRQRRR</td>
<td>GEPHKFKREW</td>
</tr>
<tr>
<td>Tat-INDIP-Mix</td>
<td>Tat 47-57</td>
<td>INSIG 152-161 (Mix)</td>
</tr>
<tr>
<td><em>(scrambled peptide)</em></td>
<td>YGRKKRRQRRR</td>
<td>RWGHRKKPFE</td>
</tr>
<tr>
<td>Tat-INDIP-2R</td>
<td>Tat 47-57</td>
<td>INSIG 152-161 (K-R)</td>
</tr>
<tr>
<td><em>(mutated peptide)</em></td>
<td>YGRKKRRQRRR</td>
<td>GEPHRFRREW</td>
</tr>
</tbody>
</table>
FIGURE 4-2. Development of the INDIP peptide.

The mechanism by which SREBP1 is activated following insig1 degradation (A) and the primary sequence of Tat-INDIP peptide and other control peptides (B) is illustrated. A, SREBP1/SCAP complex normally resides in the endoplasmic reticulum (ER) membrane with their inhibitor binding partner insig1. When cholesterol level is low or when NR2BRs are stimulated, insig1 becomes ubiquinated which triggers its degradation by the proteasome. Non-insig1-bound SREBP1/SCAP complex then travel to the Golgi apparatus where SREBP1 is truncated to release the active N-terminal fragment (nt-SREBP1) that translocates to the nucleus to carry our transcriptional activity. B, To inhibit NR2BR-to-SREBP1 neuronal death signaling, we developed a novel therapeutic peptide Tat-INDIP. The former Tat sequence confer membrane permeability to facilitate its administration in vivo, and the later INDIP sequence resembles the amino acid sequence flanking the ubiquitination sites K156 and K158 of insig1. Two control peptide, (1) Tat-INDIP-Mix where the INDIP sequence is scrambled, and (2) Tat-INDIP-2R (or Tat-INDIP-K>R) where the K156 and K158 is mutated from lysine-residues into arginine-residues is also developed.
FIGURE 4-3. Tat-INDIP protects the brain against insig1 degradation and SREBP1 activation.

A

I.V. Injection
-Occlusion (Ischemia)-
-Reperfusion
-22.5 hours

B

Hemisphere

Saline  Indip  Indip[K>R]

Insig-1

C

Insig-1 ratio Occ/Cnt

Saline  Indip  Indip[K>R]
FIGURE 4-3. Tat-INDIP protects the brain against insig1 degradation and SREBP1 activation.

D

<table>
<thead>
<tr>
<th></th>
<th>Saline Cont</th>
<th>Saline Occl</th>
<th>INDI Cont</th>
<th>INDI Occl</th>
</tr>
</thead>
<tbody>
<tr>
<td>nt-SREBP1</td>
<td><img src="D1.png" alt="Image" /></td>
<td><img src="D2.png" alt="Image" /></td>
<td><img src="D3.png" alt="Image" /></td>
<td><img src="D4.png" alt="Image" /></td>
</tr>
<tr>
<td>NeuN</td>
<td><img src="D5.png" alt="Image" /></td>
<td><img src="D6.png" alt="Image" /></td>
<td><img src="D7.png" alt="Image" /></td>
<td><img src="D8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

E

- **nt-SREBP1/NeuN**

<table>
<thead>
<tr>
<th></th>
<th>Cntl Saline</th>
<th>Occ Saline</th>
<th>Cntl Indip</th>
<th>Occ Indip</th>
</tr>
</thead>
<tbody>
<tr>
<td>nt-SREBP1/NeuN</td>
<td><img src="E1.png" alt="Image" /></td>
<td><img src="E2.png" alt="Image" /></td>
<td><img src="E3.png" alt="Image" /></td>
<td><img src="E4.png" alt="Image" /></td>
</tr>
</tbody>
</table>
FIGURE 4-3. Tat-INDIP protects the brain against insig1 degradation and SREBP1 activation.

Focal ischemic stroke induced prominent NR2BR-dependent insig1 degradation, leading to SREBP1 activation. To test the hypothesis that NR2BR-to-SREBP1 signaling may play a role in excitotoxic neuronal death following stroke, rats were subjected to 90-min middle cerebral arterial occlusion (MCAO) using the intraluminal suture-insertion technique. Each rat was euthanized to allow collection of brain tissue from the motor and sensory cortex at 3 h or 1 day following ischemia to allow western blot analysis of insig1 degradation and SREBP1 activation, respectively. Following tissue collection, each brain slice was stained with TTC to ensure that the tissues were collected from the appropriate occluded area (Occl), versus the contralateral control area (Cont). Tat-INDIP (8 mg/kg, i.v.; 45 min prior to MCAO) significantly prevented insig1 degradation and SREBP1 activation (n=3 per group).

A, timeline. B and C, western blot: representative figure (B) and summarized data (C) for insig1 protein level. D and E, western blot: representative figure (D) and summarized data (E) for the protein level of active N-terminus of SREBP1 (nt-SREBP1).

Data is expressed as mean ± sem. n=3 for all treatment and control groups.

Statistical Analysis:

C, One Way ANOVA detected significant effect of treatment on the insig1 level (P<0.01). Post-hoc multiple comparison procedures using the Holm-Sidak method found significant increases in insig1 level (*P<0.05).

E, One Way ANOVA detected significant effect of treatment on the level of active SREBP1 N-terminus fragment (nt-SREBP1) (P<0.01). Post-hoc multiple comparison procedures using the Holm-Sidak method found significant increases in nt-SREBP1 level (**P<0.01).
FIGURE 4-4. Tat-INDIP protects the rat against neurological deficits following focal ischemic stroke.
FIGURE 4-4. Tat-INDIP protects the rat against neurological deficits following focal ischemic stroke.

Focal ischemic stroke induced prominent NR2BR-dependent insig1 degradation, leading to SREBP1 activation. To test the hypothesis that NR2BR-to-SREBP1 signaling may play a role in excitotoxic neuronal death following stroke, rats were subjected to 90-min middle cerebral arterial occlusion (MCAO) using the intraluminal suture-insertion technique. Their neurological deficits were examined immediately before the rats were euthanized 24 h post-ischemia. Tat-INDIP (8 mg/kg, i.v.; 45 min prior to MCAO; n=9) significantly improved 24 h post-stroke outcome, compared to saline control (i.v.; 45 min prior to MCAO; n=9).

Data is expressed as mean ± sem.

Statistical Analysis:

Two Way Repeated Measures ANOVA (with time repetition) detected significant effect of Tat-INDIP on stroke outcome (F=5.414 and P=0.031), and also on the recovery of neurological outcome over time (F=5.232 and P=0.035). There was in general no significant recovery from stroke over time (F=3.689 and P=0.072). Post-hoc pairwise multiple comparisons using the Holm-Sidak method confirmed significant neuroprotection by Tat-INDIP compared to saline (t=2.310 and P=0.032), especially when tested 24 h post-stroke (t=3.304 and P=0.002) and not 1 h post-stroke (t=0.566 and P=5.75). Likewise, there was a significant improvement in stroke outcome over time in the Tat-INDIP-treated animals (t=3.057 and P=0.007) but not in the saline-treated animals (t=0.253 and P=0.803).
FIGURE 4-5. Tat-INDIP protects the rat brain against cerebral infarction following focal ischemic stroke.

A

I.V. Injection
+45 min

Occlusion
(Ischemia)

Reperfusion
+1.5 hrs

Sacrifice Animal
+24 hrs

45 mins
1.5 hours
22.5 hours

B

Saline
Tat-INDIP
Tat-Scrambled

Infarct Area (mm^2)

Distance from Bregma (mm)

C

Infarct Volume (% relative to control)

Saline
Tat-INDIP
Tat-Scrambled

*
FIGURE 4-5. Tat-INDIP protects the rat brain against cerebral infarction following focal ischemic stroke.
FIGURE 4-5. Tat-INDIP protects the rat brain against cerebral infarction following focal ischemic stroke.

Focal ischemic stroke induced prominent NR2BR-dependent insig1 degradation, leading to SREBP1 activation. To test the hypothesis that NR2BR-to-SREBP1 signaling may play a role in excitotoxic neuronal death following stroke, rats were subjected to 90-min middle cerebral arterial occlusion (MCAO) using the intraluminal suture-insertion technique, and euthanized 24 h post-ischemia to allow histological measurement of cerebral infarction. Tat-INDIP (8 mg/kg, i.v.; 45 min prior to MCAO; n=9) but not the scrambled control peptide Tat-INDIP-Mix (8 mg/kg, i.v.; 45 min prior to MCAO; n=6) significantly decreased cerebral infarction, compared to saline control (i.v.; 45 min prior to MCAO; n=9).


Data is expressed as mean ± sem.

B, Two Way Repeated Measures ANOVA detected significant effect of treatment on infarct areas (F=2.252 and P=0.128), and also significant differences in infarct area across the brain (F=59.416 and P<0.001). There was no significant effect of the treatments on the pattern of infarction across the brain (F=1.169 and P=0.319). Post-hoc multiple comparison procedures using the Holm-Sidak method did not find significant differences between treatment groups.

C, One Way ANOVA detected significant effect of treatment on the total infarct volume (F=3.816 and P=0.039). Post-hoc multiple comparison procedures using the Holm-Sidak method confirmed significant neuroprotection by Tat-INDIP (t=2.751 and *P=0.036).
FIGURE 4-6. Tat-INDIP post-stroke treatment improved neurological outcome over the first week following focal ischemic stroke.
FIGURE 4-6. Tat-INDIP post-stroke treatment improved neurological outcome over the first week following focal ischemic stroke.

To investigate pre-clinically whether Tat-INDIP may be an effective post-stroke treatment and whether such neuroprotection would be long lasting, rats were subjected to 90-min middle cerebral arterial occlusion (MCAO) using the intraluminal suture-insertion technique. Their neurological deficits were examined immediately, 24 h post-ischemia, and then every 48 h until the rat is euthanized one-week later. Tat-INDIP (8 mg/kg, i.v.; 2 h post-MCAO onset; n=8) improved neurological outcome over the one-week observation period following focal ischemic stroke, compared to saline control (i.v.; 2 h post-MCAO onset; n=8).

Data is expressed as mean ± sem.

Two Way Repeated Measures ANOVA detected significant effect of treatment on the neurological outcome (F=6.677 and P=0.006). In general, there was significant recovery in neurological performance over time (F=25.662 and P<0.001), and this was independent of treatment administered (F=1.885 and P=0.074). However, post-hoc multiple comparison procedures using the Holm-Sidak method failed to detect significant neuroprotection between treatment groups (t=1.517 and P=0.145).
FIGURE 4-7. Tat-INDIP post-stroke treatment attenuates delayed SREBP1 activation in the rat brain one week following focal ischemic stroke.
FIGURE 4-7. Tat-INDIP post-stroke treatment attenuates delayed SREBP1 activation in the rat brain one week following focal ischemic stroke.

B

Tat-INDIP
FIGURE 4-7. Tat-INDIP post-stroke treatment attenuates delayed SREBP1 activation in the rat brain one week following focal ischemic stroke.

To investigate pre-clinically whether Tat-INDIP may be an effective post-stroke treatment and whether such neuroprotection would be long lasting, rats were subjected to 90-min middle cerebral arterial occlusion (MCAO) using the intraluminal suture-insertion technique. One week following MCAO, rats were euthanized and paraformaldehyde-fixed to allow immunofluorescent analysis of SREBP1 activity in the brain. Representative images were taken from the striatum area of the brain. A, Surprisingly, SREBP1 activity, as evident by the prominent nuclear localization, remained strong even one week post-MCAO. This suggested that SREBP1 activation can be very long lasting post-stroke, and that therapeutics that act by targeting SREBP1 may have a very wide therapeutic window. B, Tat-INDIP (8 mg/kg, i.v.; 2 h post-MCAO onset; n=8) profoundly reduced one-week delayed SREBP1 activation (ie. nuclear localization) in the rat brain following focal ischemic stroke, compared to saline control (i.v.; 2 h post-MCAO onset; n=8).

Scale bar = 50μm
FIGURE 4-8. Tat-INDIP post-stroke treatment protects the rat brain against delayed neuronal death one week following focal ischemic stroke.

A

Occlusion (Ischemia) 1.5 hours Reperfusion +1.5 hrs I.V. Injection +2 hrs Sacrifice Animal +7 days

B

Fluorolade-positive Area (mm²)

Distance from Bregma (mm)

Saline  Indip  Indip(K-R)

C

Fluorolade-positive volume (mm³)

Saline  Indip  Indip(K-R)
FIGURE 4-8. Tat-INDIP post-stroke treatment protects the rat brain against delayed neuronal death one week following focal ischemic stroke.
FIGURE 4-8. Tat-INDIP post-stroke treatment protects the rat brain against delayed neuronal death one week following focal ischemic stroke.

To investigate pre-clinically whether Tat-INDIP may be an effective post-stroke treatment and whether such neuroprotection would be long lasting, rats were subjected to 90-min middle cerebral arterial occlusion (MCAO) using the intraluminal suture-insertion technique. One week following MCAO, rats were euthanized and paraformaldehyde-fixed to allow Fluoro-Jade B staining and analysis of degenerating neurons. Evidently, neurodegeneration remained prominent up to one week post-MCAO, and Tat-INDIP (8 mg/kg, i.v.; 2 h post-MCAO onset; n=8), but not the mutant control peptide Tat-INDIP-K>R (8 mg/kg, i.v.; 2 h post-MCAO onset; n=7), significantly reduced the area (B) and volume (C) of one-week delayed neuronal death in the rat brain following focal ischemic stroke, compared to saline control (i.v.; 2 h post-MCAO onset; n=8).


Data is expressed as mean ± sem.

B, Two Way Repeated Measures ANOVA detected significant effect of treatment on infarct areas (F=4.126 and P=0.032), and also significant differences in infarct area across the brain (F=41.034 and P<0.001). In addition, there was a significant effect of the treatments on the pattern of infarction across the brain (F=2.247 and P=0.032). Post-hoc multiple comparison procedures using the Holm-Sidak method confirmed significant reduction in infarct area at -0.4mm from bregma by Tat-INDIP, compared to saline control (t=3.333 and P=0.003) and to Tat-INDIP(K>R) (t=3.585 and 0.002).
C, One Way ANOVA detected significant effect of treatment on the total infarct volume (F=4.126 and P=0.032). Post-hoc multiple comparison procedures using the Holm-Sidak method confirmed significant neuroprotection by Tat-INDIP compared to the control peptide Tat-INDIP(K>R) (t=2.761 and *P=0.036).
CHAPTER 5:

NOVEL METHOD FOR PROTEIN KNOCKDOWN

5.1 Specific Background

5.1.1 Biodiversity from DNA to protein

The human genome project identifies approximately 20,000-25,000 genes in the human DNA. Each of these genes can be transcribed into a pre-mRNA, which in turn can be truncated into one more mRNA splice variants. The final post-splicing mRNA products can each be translated into a specific protein that is further diversified by alternative protein folding, post-translational modifications, and protein-to-protein interactions. This exponential amplification of gene-product diversity from the 20,000-25,000 genes to the many fold higher number of mRNA splice variants, and finally to the orders of magnitude higher diversities of protein forms, dictates the biology, the physiology, and the pathology of cells, organs, and the whole body.

5.1.2 DNA knockout, RNA knockdown, and limitations

The introduction of experimental techniques to nullify gene functions, such as DNA knockout in genetic engineered mice and mRNA knockdown by introducing siRNA, has allowed scientists to understand the functions of specific genes, and more importantly, it has led to the discoveries of many genes required for the manifestation of human diseases. For instance, as discussed in Chapter 1.4, DAPK1 is thought be required for neuronal death following stroke, based on the recent evidence that DNA knockout mice lacking DAPK1 were resistant to
ischemic stroke damage (Tu et al., 2010). Likewise, knocking down the mRNA for PSD95 (Sattler et al., 1999) and SREBP1 (Taghibiglou et al., 2009) in cultured cortical neurons confers neuroprotection against excitotoxic NMDAR stimulation, thus suggesting for the first time that these proteins are death signaling proteins downstream of NR2BRs. Undoubtedly, and as evident from the Nobel Prizes in Physiology and Medicine given to Mario R Capecchi, Sir Martin J Evans, and Oliver Smithies in 2007 for “their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells” and to Andrew Z Fire and Craig C Mello in 2008 for “their discovery of RNA interference - gene silencing by double-stranded RNA”, these techniques have revolutionized modern biomedical research, to say the least (refer to http://nobelprize.org).

The primary objective of the work presented here is to further build onto the expectations and improve on the limitations associated with these techniques for disrupting the stable expression of gene-products. Some of the major limitations associated with DNA knockout and mRNA knockdown techniques are describe here. First, given the amplification of diversity from genes to gene products (see Chapter 5.1.1), the present techniques discussed above are far from specific enough to study the detailed functions of specific mRNA splice-variants and proteins. DNA knockout normally knockouts all mRNA splice variants and protein variants, thus preventing investigation of a specific protein variant. This limitation also goes for siRNA-based mRNA knockdown. Selective knockdown of a mRNA splice-variant but not the pre-mRNA is challenging, and in most cases not possible. Second, many proteins have a very long half-life, remaining stably expressed even after conditional DNA-knockout or mRNA knockdown. Because disruption of genetic information processing from birth (as in conventional DNA-knockout mice) makes it hard to discern whether a specific gene is of developmental importance or of functional importance, post-developmental
conditional DNA-knockout or mRNA knockdown is usually required when studying protein function. However, these techniques are often limited to the investigation of proteins with a high turnover rate (or short half-life), as it becomes cumbersome to study functions of proteins with long half-life by comparing phenotypes of test subjects before and after gene-disruption. Third, despite the past attempts to develop gene-therapy, it remains by and large clinically impractical to knockout DNA or knockdown mRNA in human subjects (Castanotto and Rossi, 2009). This limitation in essence delays the translation of research findings discovered using these techniques to clinical use (Castanotto and Rossi, 2009).

5.1.3 Ubiquitin as molecular determinant for protein degradation

Ubiquitin is a 76 amino-acid long protein that is ubiquitously expressed in all cells, and its sequence is highly evolutionarily conserved between bacteria and human (Finley and Chau, 1991; Glickman and Ciechanover, 2002). Ubiquitin can be covalently conjugated to almost any proteins in the cell, via a process called ubiquitination, by key enzymes dedicated for this process: the obligatory ubiquitin activator (the E1 enzyme), the ubiquitin conjugators (the E2 enzymes), and the ubiquitin ligases (the E3 enzymes) (Finley and Chau, 1991; Glickman and Ciechanover, 2002). Research in the past few decades strongly suggests that ubiquitination of a protein is required for its degradation, either by the lysosome (for 20% of proteins) or the proteasome (for 80% of proteins) (Ciechanover et al., 1980a; Ciechanover et al., 1980b; Finley and Chau, 1991; Glickman and Ciechanover, 2002; Hershko et al., 1980; Hershko et al., 1979). That being said, it should be noted that ubiquitination has many other functions in addition to protein degradation, and these include the endocytosis of membrane proteins, the nuclear translocation of proteins, the functional activation of proteins, and so on (Ikeda and Dikic, 2008; Lelouard et al., 2002; Mukhopadhyay and Riezman, 2007; Trotman et al., 2007).
The specific effect of ubiquitination on a protein largely depends on which site on the protein is ubiquitinated, and more importantly, which site on the ubiquitin is being further ubiquitinated by a process known as “polyubiquitination” (Chau et al., 1989; Ikeda and Dikic, 2008; Mukhopadhyay and Riezman, 2007).

5.1.4 Degradation of non-tagged proteins

Even though ubiquitination is required for the regulated degradation of many otherwise-stable proteins (Finley and Chau, 1991; Glickman and Ciechanover, 2002), and also required for the constitutive degradation of short-lived proteins (Ciechanover et al., 1984; Finley et al., 1984; Glickman and Ciechanover, 2002), proteasome-mediated degradation also occurs in proteins without a clear ubiquitination site (Glickman and Ciechanover, 2002). Moreover, it remains puzzling how ubiquitination can occur for some proteins whose ubiquitination sites appear inaccessible to the ubiquitinating enzymes E1, E2 and E3. These can include, for instance, intra-membrane proteins that may be partly or wholly submerged in the plasma membrane or organelle membrane. The dogmatic standpoint that ubiquitination is essential for protein degradation came in large from early findings that mutant ts85 cell lines with temperature-sensitive E1 are defective in protein degradation under non-permissive temperature (Finley et al., 1984; Glickman and Ciechanover, 2002). However, these experiments do not defeat the possibility that some proteins do not need to be ubiquitin-tagged per se, but rather, ubiquitination of other proteins may facilitate the degradation these non-tagged proteins. In other words, even though it is generally thought that ubiquitination is a highly specific process (Finley and Chau, 1991), such that only proteins tagged with ubiquitin will be degraded, it has not been tested if non-tagged protein may be degraded due to ubiquitination of another protein. The first set of evidence to support the possibility that non-tagged protein can also be
degraded through protein-to-protein interaction with an ubiquitin-tagged protein is presented here.

5.2 Results

5.2.1 Degradation of linear ubiquitin-linked protein in human cells

In modeling ubiquitination and protein degradation in the human cell line HEK 293, the cellular stability of Homer1b, a well-characterized constitutively expressed protein (Beneken et al., 2000; Brakeman et al., 1997; Tu et al., 1998), expressed with and without a linear N-terminal ubiquitin-tag was measured by means of western blot analysis (FIGURE 5-1). Consistent with Homer1b being a stable protein, a prominent band reflecting non-tagged Homer1b was detected by western blot. In marked contrast, almost no Ubiquitin-tagged Homer1b appeared, except in the presence of the proteasome inhibitor epoxomicin, in which equal level of Homer1b and Ubiquitin-Homer1b appeared. Notably, with epoxomicin, Ubiquitin-Homer1b appeared to have the same molecular weight as Homer1b, suggesting that most of the ubiquitin has been actively removed by de-ubiquitinating enzymes (DUBs) (Bachmair and Varshavsky, 1989). This triggered the possibility that degradation did not occur by linear N-terminal ubiquitination per se, but rather by de-ubiquitination followed by re-ubiquitination of the internal lysine residues. To confirmed that linear N-terminal ubiquitination directly induced protein degradation, the mutant protein Ubiquitin(G76V)-Homer1b was constructed to confer resistance to the DUBs (Johnson et al., 1992; Johnson et al., 1995). As expected, Ubiquitin(G76V)-Homer1b also actively degraded, and its rescue by the proteasomal inhibitor epoxomicin demonstrated it was indeed resistant to DUBs as evident from its higher molecular weight compared to non-tagged Homer1b (see FIGURE 5-1).
5.2.2 Facilitated degradation of non-tagged proteins

Based on the model system in HEK cell line, the possibility that ubiquitin-tagged protein can facilitate the degradation of non-tagged proteins was tested by expressing either Homer1b alone, Ubiquitin(G76V)-Homer1b alone, or both Homer1b and Ubiquitin(G76V)-Homer1b (FIGURE 5-2). Again, Homer1b demonstrated little evidence of degradation, relative to Ubiquitin(G76V)-Homer1b that actively underwent proteasomal degradation. Surprisingly, co-expression of these two proteins resulted in the facilitated degradation of Homer1b, rather than just Ubiquitin(G76V)-Homer1b. Like the degradation of Ubiquitin(G76V)-Homer1b, the facilitated degradation of Homer1b was also rescued by the proteasome inhibitor epoxomicin. Notably, facilitated degradation of Homer1b could not be due to increased cellular ubiquitin resulting from de-ubiquitination of Ubiquitin(G76V)-Homer1b, because as shown earlier, this recombinant mutant is resistant to DUBs (see FIGURE 5-1). To the best of our knowledge, this was the first experimental data demonstrating that non-tagged protein could be degraded by the co-presence of an ubiquitin-tagged protein. Moreover, because Homer1b could interact with Ubiquitin(G76V)-Homer1b through direct protein-to-protein interaction (Xiao et al., 1998), this finding was consistent with the notion that facilitated degradation of non-tagged proteins required protein-to-protein interaction with ubiquitin-tagged proteins.

We next sought to investigate whether Ubiquitin(G76V)-Homer1b may facilitate the degradation of an unrelated protein mGFP in its native form, when it is tagged with the Homer1b sequence, and when it is tagged with the Homer1a sequence (FIGURE 5-3). Co-expression of Homer1b did not affect the stability of GFP-Homer1b, compared to its sole-expression. In marked contrast, co-expression of Ubiquitin(G76V)-Homer1b facilitated the degradation of GFP-Homer1b, and this facilitated degradation could be rescued by the proteasome inhibitor epoxomicin. Consistent with the requirement for protein-to-protein
interaction and given that Homer1b do not interact with Homer1a (Xiao et al., 1998), Ubiquitin(G76V)-Homer1b did not facilitate the degradation of either mGFP or mGFP-Homer1a. Importantly, because Homer1a is a mRNA splice variant of Homer1b (Xiao et al., 1998), that these two proteins shared the same pre-mRNA and same DNA gene, the selective degradation of GFP-Homer1b but not GFP-Homer1a demonstrated the strength of this facilitated protein degradation over conventional techniques for disruption of gene-products, namely DNA knockout and mRNA knockdown (see Chapter 5.1.2).

The various characteristics of this “facilitated protein degradation” or “direct protein knockdown” was thereafter further investigated. First, in testing whether this facilitated degradation was all-or-none or dose-dependent, we increased the gene-dose for the expression of Ubiquitin(G76V)-Homer1b in the above mentioned preparation, and found improved facilitated degradation of GFP-Homer1b as the dose of Ubiquitin(G76V)-Homer1b increased (FIGURE 5-4). Second, to further confirm that this facilitated protein degradation required directed protein-to-protein interaction, we co-expressed GFP-Homer1b with either native Homer1b as a negative control, Ubiquitin(G76V)-Homer1b as a positive control, Ubiquitin(G76V)-Homer1b(1-120) that lacks the C-terminus for protein-protein interaction (Xiao et al., 1998), and Ubiquitin(G76V)-Homer1b(188-354) that lacks the N-terminus (FIGURE 5-5). As predicted by the requirement for protein-protein interaction, Ubiquitin(G76V)-Homer1b(188-354) retained the efficacy to induce facilitated degradation of GFP-Homer1b, whereas Ubiquitin(G76V)-Homer1b(1-120) completely lost the ability to facilitate the degradation of GFP-Homer1b. Third, because Homer1b was known to interact with the C-terminus of mGluR5 (mGluR5-CT) with a specific requirement for the amino-acid-sequence xPPxxFx (Beneken et al., 2000; Brakeman et al., 1997; Tu et al., 1998), we tested the effect of Ubiquitin(G76V)-mGluR5-CT-mGFP, with and without the point-mutation at the
xPPxxFx site, on mGFP-Homer1b (FIGURE 5-6). As expected, Ubiquitin(G76V)-mGluR5-CT-mGFP, in addition to its own degradation, facilitated the degradation mGFP-Homer1b; in contrast, the non-tagged mGluR5-CT-mGFP was not degraded, and also did not facilitate the degradation of mGFP-Homer1b. Impressively, a single point-mutation (P>Q) of the protein-protein interaction site from xPPxxFx to xPQxxFx, previously shown to be required for Homer1b-to-mGluR5 interaction (Tu et al., 1998), resulting in Ubiquitin(G76V)-mGluR5-CT(P>Q)-mGFP, partly rescued the facilitated degradation of mGFP-Homer1b.

5.2.3 Deciphering the ubiquitin for optimal protein degradation

Given our discovery that non-tagged proteins may be knocked down through protein-protein interaction with ubiquitin-tagged proteins, there are 3 possible ways to translate this finding to in vivo animal research and clinical use. First, one can directly infect animal tissue in vivo with a virus carrying the plasmid for over-expressing the ubiquitin-tagged protein. This is essentially the same as the current approach for infecting animal tissues with siRNA (reviewed in (Castanotto and Rossi, 2009)), but with the added benefit that the targeted protein rather than its mRNA will be knocked down. Second, one can purify a recombinant Tat-linked version of the ubiquitin-tagged protein from a protein-expressing bacterial system (Brooks et al., 2005). The “Tat” sequence is essentially the MTD derived from the HIV1 Tat protein that confers linked protein membrane and BBB permeability (see Chapter 1.6.3). These biologics will be much easier to deliver in vivo than are viruses designed for gene therapy, and given the clinical safety of Tat-linked peptides in clinical studies (see Chapter 1.6.3), these biologics will be directly applicable for clinical use. Third, one can synthesize the whole TAT-linked Ubiquitin-tagged protein as a synthetic peptide given that the total length does not exceed the maximum possible for peptide synthesis (up to ~120 amino acids long for some facilities),
which largely depends on the individual facility. This raises opportunity for a broad range of scientists with little knowledge of virus construction and molecular biology to custom-design their own peptide to knockdown proteins in their particular study.

Having said the above, there remains much room for optimization before this technology can become widely applicable. The major obstacle would be the difficulty and cost for producing these Tat-linked ubiquitin-tagged biologics, be it recombinant proteins or synthetic peptides. The large-scale expression and purification of recombinant proteins can be difficult, especially given the size of each Tat-linked ubiquitin-tagged biologic by the present design. In addition, most peptide-synthesizing facilities cannot synthesize peptides longer than 60 amino-acids long, and for the facilities that do, the cost can be substantial for even very small quantities (reference: personal communication with commercial peptide synthesizing factories). Therefore, optimizing the present design by effectively reducing the length of a given Tat-linked ubiquitin-tagged biologic for direct protein knockdown in in vivo animal experiments or for clinical treatment of diseases is of great importance.

Although the degradation of Ubiquitin-tagged peptide would be hard to study due to its small size, the facilitated degradation of a large protein by protein-protein interaction with an Ubiquitin-tagged peptide could be easily measured by western blot. Given that Ubiquitin(G76V)-mGluR5-CT49 actively knocked down Homer1b in the above mentioned experiment, we sought to investigate whether this effect remains following truncation of this protein, leaving only the 8 amino acids (sequence: TPPSPFRDS) containing the essential sequence xPPxxFx required for mGluR5-to-Homer1b interaction (Beneken et al., 2000; Tu et al., 1998) (FIGURE 5-7). We found that Ubiquitin(G76V)-TPPSPFRDS indeed knocked down Homer1b, compared to the control non-tagged peptide M-TPPSPFRDS.

We next sought to decipher the ubiquitin amino-acid sequence for the sites essential for
protein degradation. Although each ubiquitin molecule contains 7 internal lysine residues that can be further ubiquitinated in a process known as ‘polyubiquitination’ (Chau et al., 1989), previous studies in yeast cells (S. cerevisiae) suggested that only the K29, K48, and K63 sites are substrates for polyubiquitination, and of these three sites, only K29 and K48 sites putatively participate in protein degradation (Johnson et al., 1995). To pinpoint which of the 7 lysine-residues are the essential sites for protein degradation in mammalian cells, we did a more extensively screening in which a series of mutated Ubiquitin(G76V)-Homer1b plasmids were constructed to have each one of the 7 internal lysine residues on the ubiquitin moiety converted into an arginine residue, rendering the site resistant to polyubiquitination (FIGURE 5-8, A). Overexpression of these mutant Ubiquitin(G76V)-Homer1b in HEK 293 cells revealed that, consistent with predictions based on previous studies (Johnson et al., 1995), only the K29 and the K48 sites, but not the K6, the K11, the K27, the K33, and the K63 sites, contributed to protein degradation (FIGURE 5-8, B and C). Impressively, double-mutation of both K29 and K48, but not single mutation of each site, resulted in complete rescue of protein degradation (FIGURE 5-8, D and E). This raised the intriguing possibility that either one of the K29 and the K48 sites were sufficient for protein degradation.

Based on these findings, we did a series of truncation to the ubiquitin moiety on Ubiquitin(G76V)-Homer1b around the K29 and the K48 sites, from the N-terminus, from the C-terminus, or from both termini, and made the following observations. First, N-terminal truncation of the first 9 amino acids (resulting in Ubiquitin:10-76) strongly suppressed Ubiquitin(G76V)-Homer1b protein degradation, whereas further truncation of the next 11-20 amino acids around the K29 polyubiquitination site (resulting in Ubiquitin:21-76 and Ubiquitin:30-76) resulted in complete suppression of protein degradation (FIGURE 5-9). Nevertheless, and somewhat surprisingly, protein degradation activity was substantially
rescued after truncation of first 36 amino acids (resulting in Ubiquitin: 37-76), only to become completely suppressed with further truncation past the essential K48 polyubiquitination site (Ubiquitin:49-76 and Ubiquitin:64-76). Second, C-terminal truncation of the last 19 amino acids (resulting in Ubiquitin:1-57) only partially suppressed Ubiquitin(G76V)-Homer1b protein degradation (FIGURE 5-10). Moreover, and again surprisingly, this partial suppression was further alleviated by further truncation from the C-terminus (resulting in Ubiquitin:1-45 and Ubiquitin:1-38). Third, based on the above N-terminus and C-terminus truncation findings, we further made double-truncations of both termini (FIGURE 5-11). Double-termini truncations of the ubiquitin moiety on Ubiquitin(G76V)-Homer1b in general led to much poorer degradation, compared to the full length protein or the N-terminal truncated protein Ubiquitin(37-76; G76V)-Homer1b. Nonetheless, there remained evidently more degradation than the non-tagged Homer1b. These results demonstrated the efficacy of truncated ubiquitin-moieties in mediating protein degradation, and suggested that shorter ubiquitin-moieties may replace the full-length ubiquitin in the design and production of biologics for protein-knockdown.
FIGURE 5-1. Degradation of N-terminal ubiquitin-tagged protein and resistance to DUB.

A Ubiquitin-fusion proteins are prone to DUB, resulting in impaired protein degradation.

B G76V mutant is resistant to DUB.
FIGURE 5-1. Degradation of N-terminal ubiquitin-tagged protein and resistance to DUB.

C

Homer1b

β-Tubulin

+Epoxomicin

Ubi-H1b

H1b  GG  GV

Ubi-H1b

H1b  GG  GV
FIGURE 5-1. Degradation of N-terminal ubiquitin-tagged protein and resistance to DUB.

A. N-terminal ubiquitin-tagged proteins are actively un-tagged by de-ubiquitinating enzymes (DUBs) upon expression, resulting in compromised degradation and increased cellular ubiquitin level. B. Mutation of the last amino-acid residue of ubiquitin from glycine into valine prevents DUB reactions, and confers uncompromised proteasomal degradation. C. Western blot: Expression of Homer1b (H1b) in HEK 293 cells was prominent and stable, whereas expressions of Ubiquitin-Homer1b (wild-type: GG) and its DUB-resistant mutant (mutant: GV) were short-lasting due to active proteasomal degradation. Inhibition of proteasome by epoxomicin (1µM) revealed that Ubiquitin-Homer1b was a substrate for DUB, which actively un-tagged the protein to give a similar molecular weight as native Homer1b. In contrast, Ubiquitin(G76V)-Homer1b was not a substrate for DUB, having a higher molecular weight than Homer1b due to retention of its ubiquitin-tag. β-tubulin served as a loading control in this experiment.
FIGURE 5-2. Ubiquitin-tagged protein facilitates the degradation of non-tagged proteins.

A

Homer1b

Homer1b

UBI(v)-H1b

Homer1b

+Epoxomicin

B

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<th>Homer1b</th>
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Homer1b

β-Tubulin
FIGURE 5-2. Ubiquitin-tagged protein facilitates the degradation of non-tagged proteins.
FIGURE 5-2. Ubiquitin-tagged protein facilitates the degradation of non-tagged proteins.

A. Homer1b may interact with other Homer1b molecules through direct protein-protein interaction, and as such, Ubiquitin-Homer1b or Ubiquitin(G76V)-Homer1b may interact with non-tagged native Homer1b. In the present study, we test the hypothesis whether Ubiquitin-tagged Homer1b may facilitate the degradation of non-tagged Homer1b, and whether this indirect facilitated degradation may be rescued by inhibitor of the proteasome.

B. Western blot: Ubiquitin(G76V)-Homer1b degraded abruptly upon expression in HEK 293 cells (n=4), compared to non-tagged native Homer1b (n=4). Moreover, co-expression of these two proteins resulted in degradation of not only Ubiquitin(G76V)-Homer1b, but also the non-tagged native Homer1b (n=4). The proteasomal inhibitor epoxomicin not only rescued Ubiquitin(G76V)-Homer1b degradation, but also rescued the indirect facilitated degradation of non-tagged Homer1b (n=3 per group).

C and D, summarized data from the western blots. β-tubulin served as a loading control in this experiment.

Abbreviations: H1b, Homer1b; UBI(v)-H1b, Ubiquitin(G76V)-Homer1b.

Statistical Analysis:

C, Two way ANOVA detected significant differences in Homer1b level due to ubiquitination (Homer1b versus Ubi-Homer1b; F=177.0 and P<0.0001), and also due to co-expression of Ubi-Homer1b (knockdown of Homer1b by Ubi-Homer1b; F=68.44 and P<0.0001). Post-hoc multiple comparisons by the Bonferroni method confirmed that Ubi-Homer1b significantly knocked down Homer1b (t=11.82 and *P<0.0001), whereas native Homer1b had no effect on Ubi-Homer1b protein level (t=0.1161 and P>0.05).

D, Two way ANOVA detected no significant difference in protein level due to ubiquitination
(Homer1b versus Ubi-Homer1b; F=1.765 and P=0.2087), and also no difference due to co-expression of Ubi-Homer1b (knockdown of Homer1b by Ubi-Homer1b; F=0.07528 and P=0.7885).
FIGURE 5-3. Ubiquitin(G76V)-Homer1b selectively knocked down GFP-Homer1b, but not GFP-Homer1a or native GFP.
FIGURE 5-3. Ubiquitin(G76V)-Homer1b selectively knocked down GFP-Homer1b, but not GFP-Homer1a or native GFP.

B

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<td>+Epoxomicin</td>
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GFP-Homer1b

β-Tubulin

C

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<th>GFP-Homer1b/Tubulin (%) of control</th>
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<tr>
<td>Homer1b</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>Ubi-Homer1b</td>
<td>100 ± 5</td>
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</table>

- Epoxomicin
+ Epoxomicin

* p < 0.05
FIGURE 5-3. Ubiquitin(G76V)-Homer1b selectively knocked down GFP-Homer1b, but not GFP-Homer1a or native GFP.

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<td>GFP</td>
<td>+</td>
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<tr>
<td></td>
<td>Homer1b</td>
<td>+</td>
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<td></td>
<td>UBI(v)-H1b</td>
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![GFP and β-Tubulin blots](image)

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<tr>
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<tr>
<td></td>
<td>Homer1b</td>
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<td></td>
<td>UBI(v)-H1b</td>
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![Homer1a and β-Tubulin blots](image)
FIGURE 5-3. Ubiquitin(G76V)-Homer1b selectively knocked down GFP-Homer1b, but not GFP-Homer1a or native GFP.

A. Even though Homer1b may interact with GFP-linked proteins containing the entire Homer1b sequence, it cannot interact with its alternative splice variant Homer1a which shared the same pre-mRNA or other unrelated protein such as native GFP. We model that facilitated protein degradation of non-tagged proteins by ubiquitin-tagged proteins requires direct protein-protein interaction. Under this model, Ubiquitin(G76V)-Homer1b may interact with GFP-Homer1b and thereby facilitating its degradation, but because it cannot interact with native GFP or GFP-Homer1a, it would have no effect on these proteins. B-E. Western Blot: Ubiquitin(G76V)-Homer1b but not native Homer1b facilitated the degradation of mGFP-Homer1b (n=3 per group), and this facilitated degradation was rescued by the proteasome inhibitor epoxomicin (1μM) (n=3 per group) (B and summarized data in C). In marked contrast, Ubiquitin(G76V)-Homer1b had no effect on degradation of native GFP (D) and Homer1a-GFP (E). β-tubulin served as a loading control in this experiment. Abbreviations: GFP, mGFP; H1b, Homer1b, GFP-H1b, mGFP-Homer1b; Ubi(v)-H1b, Ubiquitin(G76V)-Homer1b.

Data is expressed as mean ± sem.

Statistical Analysis:

C. Two way ANOVA detected significant effect of Ubi-Homer1b (F=9.400 and P=0.0035) but not of epoxomicin (F=3.176 and P=0.1000) on GFP-Homer1b expression. Also, Ubi-Homer1b made no difference to the effect of epoxomicin on GFP-Homer1b expression (F=3.418 and P=0.0669). Post-hoc multiple comparisons by the Bonferroni method found
significant knockdown of GFP-Homer1b by Ubi-Homer1b (t=3.163 and *p<0.05) but not by native Homer1b (t=0.07654 and P>0.05) or control no-treatment (t=0.0 and P>0.05).
FIGURE 5-4. Ubiquitin(G76V)-Homer1b knocks down GFP-Homer1b in a dose-dependent manner.

A

mGFP-H1b →
UBI(v)-H1b→
Homer1b→

B

+Epoxomicin

mGFP-H1b →
UBI(v)-H1b→
Homer1b→

β-tubulin
FIGURE 5. Ubiquitin(G76V)-Homer1b knocks down GFP-Homer1b in a dose-dependent manner.

Facilitated degradation of mGFP-Homer1b in HEK293 cells by co-expression of Ubiquitin(G76V)-Homer1b but not native Homer1b was augmented by increasing the gene-dose (or the executive-protein to target-protein ratio) (A), and this was largely rescued by the proteasome inhibitor epoxomicin (1μM) (B). β-tubulin served as a loading control in this experiment. Abbreviations: GFP-H1b, mGFP-Homer1b; Ubi(v)-H1b, Ubiquitin(G76V)-Homer1b.
FIGURE 5-5. Truncation of protein-protein interaction site prevented GFP-Homer1b knockdown by Ubiquitin(G76V)-Homer1b.
FIGURE 5-5. Truncation of protein-protein interaction site prevented GFP-Homer1b knockdown by Ubiquitin(G76V)-Homer1b.
FIGURE 5-5. Truncation of protein-protein interaction site prevented GFP-Homer1b knockdown by Ubiquitin(G76V)-Homer1b.
FIGURE 5-5. Truncation of protein-protein interaction site prevented GFP-Homer1b knockdown by Ubiquitin(G76V)-Homer1b.

A. Homer1b interacts with other Homer1b proteins via its C-terminus coiled-coiled domain. Based on the hypothesis that “targeted protein degradation” requires protein-protein interactions, we predict that Ubiquitin(G76V)-Homer1b and Ubiquitin(G76V)-Homer1b(188-354) with N-terminal deletion, but not Ubiquitin(G76V)-Homer1b(1-120) with C-terminal deletion, may knockdown mGFP-Homer1b through protein-protein interaction. B-E. Expression of Ubiquitin(G76V)-Homer1b and Ubiquitin(G76V)-Homer1b(188-354) (with amino-acid-residues 1-187 deleted), but not native Homer1b and Ubiquitin(G76V)-Homer1b(1-120) (with amino-acid-residues 121-354 deleted), facilitated the degradation of mGFP-Homer1b in HEK 293 cells (n=4 per group) (B, and summarized data in D), and this facilitated degradation was rescued by the proteasome inhibitor epoxomicin (1µM) (n=4 per group) (C, and summarized data in E). β-tubulin served as a loading control in this experiment. Abbreviations: mGFP-H1b, mGFP-Homer1b; Ubi(v)-H1b, Ubiquitin(G76V)-Homer1b; Ubi(v)-HNT, Ubi(v)-Homer1b(1-120), Ubiquitin(G76V)-Homer1b(1-120); Ubi(v)-HCT, Ubi(v)-Homer1b(188-354), Ubiquitin(G76V)-Homer1b(188-354).

Data is expressed as mean ± sem. ONE-WAY ANOVA p<0.001, followed by post-hoc Holm Sidak test *p<0.05

D. One way ANOVA detected significant effect of treatments on GFP-Homer1b expression (F=13.73 and P=0.0003). Post-hoc multiple comparisons by Tukey’s test found significant knockdown of GFP-Homer1b by Ubi-Homer1b (q=6.337 and *P<0.05) and Ubi-HCT (q=7.526 and *P<0.05), but not by Ubi-HNT (q=1.266 and P>0.05).

E. One way ANOVA failed to detect significant effect of treatments on GFP-Homer1b
expression (F=0.4672 and P=0.7106). Post-hoc multiple comparisons by Tukey’s test confirmed no knockdown of GFP-Homer1b by Ubi-Homer1b (q=0.3247 and P>0.05) and Ubi-HCT (q=0.9197 and P>0.05).
FIGURE 5-6. Single point-mutation of protein-protein interaction site attenuated GFP-Homer1b knockdown by Ubiquitin(G76V)-mGluR5CT49.
FIGURE 5-6. Single point-mutation of protein-protein interaction site attenuated GFP-Homer1b knockdown by Ubiquitin(G76V)-mGluR5-CT49.

A. Homer1b interacts with the carboxyl-terminus of metabotropic glutamate receptor-5 (mGluR5-CT) at the xPPxxFx site. Therefore, it should be possible to knockdown Homer1b with Ubiquitin-tagged mGluR5-CT49 (last 49 amino-acid-residues of mGluR5-CT). B. Ubiquitin(G76V)-mGluR5-CT49-GFP facilitated the degradation of mGFP-Homer1b in HEK 293 cells, and this facilitated degradation was attenuated by a single point-mutation at the xPPxxFx protein-protein interaction site to xPQxxFx (n=2). β-tubulin served as a loading control in this experiment. Abbreviations: mGluR5, metabotropic glutamate receptor-5; mGFP-H1b, mGFP-Homer1b; CT-GFP, mGluR5-CT49-GFP; UBI-CT-GFP, Ubiquitin(G76V)-mGluR5-CT49-GFP; CTmut-GFP, mGluR5-CT49(P>Q)-GFP; UBI-CTmut-GFP, Ubiquitin(G76V)-mGluR5-CT49(P>Q)-GFP.
FIGURE 5-7. Facilitated degradation of Homer1b by ubiquitin-linked peptide, Ubiquitin(G76V)-mGluR5-CT8.

A

B

Homer1b

β-Tubulin
FIGURE 5-7. Facilitated degradation of Homer1b by ubiquitin-linked peptide, Ubiquitin(G76V)-mGluR5-CT8.

C

\[
\begin{array}{cccccc}
\text{GFP-Homer1b} & + & + & + & + & + \\
\text{Homer1b} & + & - & 3x & 6x & 9x \\
\text{UBI(v)-TPPSPFRDS} & - & + & 3x & 6x & 9x \\
\end{array}
\]

\[\text{mGFP-H1b} \rightarrow\]

\[\text{Homer1b} \rightarrow\]

\[\text{β-tubulin}\]

D

\[+\text{Epoxomicin}\]

\[
\begin{array}{cccccc}
\text{GFP-Homer1b} & + & + & + & + & + \\
\text{Homer1b} & + & - & 3x & 6x & 9x \\
\text{UBI(v)-TPPSPFRDS} & - & + & 3x & 6x & 9x \\
\end{array}
\]

\[\text{mGFP-H1b} \rightarrow\]

\[\text{Homer1b} \rightarrow\]

\[\text{β-tubulin}\]
FIGURE 5-7. Facilitated degradation of Homer1b by ubiquitin-linked peptide, Ubiquitin(G76V)-mGluR5-CT8.

A. Homer1b interacts with the xPPxxFx amino-acid sequence of the mGluR5-CT. Based on our protein knockdown model, Ubiquitin(G76V)-linked to a short peptidic fragment of mGluR5-CT (mGluR5CT8 peptide sequence: TPPSPFRDS) may knock down Homer1b through direct protein-protein interaction. B. Ubiquitin(G76V)-TPPSPFRDS but not the non-tagged peptide, knocked down wild type Homer1b expressed in HEK 293 cells. C and D. Ubiquitin(G76V)-TPPSPFRDS knocked down mGFP-Homer1b expressed in HEK 293 cells across different gene-doses (C), and this protein knockdown was inhibited by the proteasome inhibitor epoxomicin (1µM) (D). β-tubulin served as a loading control in this experiment. Abbreviations: mGlu5, metabotropic glutamate receptor-5. Ubi(v)-TPPSPFRDS, Ubiquitin(G76V)-mGluR5-CT8.
FIGURE 5-8. Deciphering the polyubiquitination sites of ubiquitin via single lysine-to-arginine mutations.

A

B

C

Homer1b

β-Tubulin

Homer1b

β-Tubulin

+Epoxomicin
FIGURE 5-8. Deciphering the polyubiquitination sites of ubiquitin via single lysine-to-arginine mutations.

D

UBI(v)-Homer1b

H1b WT R29 R48 2*R

Homer1b

\beta-Tubulin

E

+Epoxomicin

UBI(v)-Homer1b

H1b WT R29 R48 2*R

Homer1b

\beta-Tubulin
FIGURE 5-8. Deciphering the polyubiquitination sites of ubiquitin via single lysine-to-arginine mutations.

A. Schematics of Ubiquitin-tagged protein showing the 7 internal lysine-residues that can be further ubiquitinated in a process known as ‘polyubiquitination’. In particular, we and others find K29 and K48 to be the 2 critical residues contributing to protein degradation post-polyubiquitination. B and C. Lysine-to-arginine mutation (K>R) on K29 and K48, but not K6, K11, K27, K33, and K63, attenuated degradation of Ubiquitin(G76V)-Homer1b in HEK 293 cells (B), and these degradation could all be rescued by the proteasome inhibitor epoxomicin (1μM) (C). Moreover, in the presence of epoxomicin, it became clear that none of the above mutations had an effect on protein expression (C). D and E. Although K29(K>R) and K48(K>R) mutations only partially attenuated protein degradation, double-mutation of these two sites completely prevented Ubiquitin(G76V)-Homer1b degradation in HEK 293 cells, comparable to non-tagged Homer1b (D). Again, with epoxomicin, it was clear that double-mutation had no effect on Ubiquitin(G76V)-Homer1b protein expression, and any differences was due to effect on protein degradation (E). β-tubulin served as a loading control in this experiment. Abbreviations: K, lysine; R, arginine; UBI(v), Ubiquitin(G76V).

A. Schematics showing N-terminal truncation of the ubiquitin moiety of Ubiquitin(G76V)-Homer1b at various positions relative to the two lysine-residues K29 and K48, polyubiquitination sites previously shown to be critical for protein degradation. B. N-terminal truncations in the ubiquitin-moiety, resulting in Ubiquitin(10-76; G76V)-Homer1b, Ubiquitin(21-76; G76V)-Homer1b, and Ubiquitin(21-76; G76V)-Homer1b, profoundly decreased degradation when these proteins were expressed in HEK 293 cells, in comparison to their full-length version Ubiquitin(1-76; G76V)-Homer1b that degraded abruptly when expressed. Further truncation, resulting in Ubiquitin(37-76; G76V)-Homer1b, resumed degradation. The final truncations beyond the K48 site, resulting in Ubiquitin(49-76; G76V)-Homer1b and Ubiquitin(64-76; G76V)-Homer1b, again inhibited degradation. C. Protein degradation was inhibited by the proteasome inhibitor epoxomicin, and this result showed that none of the mutations affected protein expression. β-tubulin served as a loading control in this experiment. Abbreviations: K, lysine; R, arginine; H1b, Homer1b, UBI(v), Ubiquitin(G76V).
FIGURE 5-10. C-terminal truncation of the ubiquitin moiety and requirement for protein degradation.

A

B

C

<table>
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<td>+Epoxomicin</td>
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FIGURE 5-10. C-terminal truncation of the ubiquitin moiety and requirement for protein degradation.

A. Schematics showing C-terminal truncation of the ubiquitin moiety of Ubiquitin(G76V)-Homer1b at various positions relative to the two lysine-residues K29 and K48, polyubiquitination sites previously shown to be critical for protein degradation. B. C-terminal truncations in the ubiquitin-moiety, resulting in Ubiquitin(1-57; G76V)-Homer1b, Ubiquitin(1-45; G76V)-Homer1b, and Ubiquitin(1-38; G76V)-Homer1b, profoundly decreased degradation when these protein are expressed in HEK 293 cells, in comparison to their full-length version Ubiquitin(1-76; G76V)-Homer1b that degrades abruptly when expressed. C. Protein degradation was inhibited by the proteasome inhibitor epoxomicin. β-tubulin served as a loading control in this experiment. Abbreviations: K, lysine; R, arginine; H1b, Homer1b, UBI(v), Ubiquitin(G76V).

A

B

C

+Epoxomicin

Homer1b

β-Tubulin

H1b 1-76 37-76 37-45 37-57 10-38 21-38 1-38

Homer1b

β-Tubulin

K29-H1b

K48-H1b

K29-H1b

K48-H1b

K48-H1b

A. Schematics showing double-termini truncation of the ubiquitin moiety of Ubiquitin(G76V)-Homer1b at various positions relative to the two lysine-residues K29 and K48, polyubiquitination sites previously shown to be critical for protein degradation.  B. Double-termini truncations of the ubiquitin moiety on Ubiquitin(G76V)-Homer1b in general led to much poorer degradation, compared to the full length protein or the N-terminal truncated protein Ubiquitin(37-76; G76V)-Homer1b.  C. Protein degradation was inhibited by the proteasome inhibitor epoxomicin. β-tubulin served as a loading control in this experiment.

Abbreviations: K, lysine; R, arginine; H1b, Homer1b, UBI(v), Ubiquitin(G76V).
CHAPTER 6: DISCUSSION

6.1 Overall Significance of Research Findings

NMDARs are important for many neuronal functions, including opposing functions like neuronal survival and death (Lai et al., 2011). Based on our current “NMDAR location and subtype” hypothesis, physiological synaptic transmission maintains neuronal survival by activating synaptic/NR2A NMDAR’s downstream NSC, whereas glutamate release due to reverse functioning of glutamate transporters during stroke stimulates extrasynaptic/NR2B NMDAR’s downstream NDC (see Chapter 1.3 for detailed discussion). Given that neuronal death function of NMDAR is an important mechanism for stroke damage, NMDAR blockers are developed and tested in human clinical studies (Boast et al., 1988; Choi, 1987a; Woodruff et al., 1987). However, because NMDARs mediate so many different neuronal functions, blocking these receptors result in undesirable side effects (Palmer, 2001; Wood and Hawkinson, 1997). Moreover, because these receptors have a neuronal survival function, blocking these receptors can exacerbate stroke outcomes (Albers et al., 2001; Lees et al., 2001). Finally, once NMDARs’ downstream death-signaling proteins are activated, including those immediately downstream in the NDC and those further downstream beyond the NDC, blocking these receptors is no longer useful (Gladstone et al., 2002; Wood and Hawkinson, 1997).

In light of the critical role of NMDARs in mediating stroke damage and the clinical failure of conventional NMDAR blockers, the central objective of the present study aims to develop novel NMDAR-based therapeutics that overcome the clinical limitations mentioned above by having a wider therapeutic time window and by eliminating neurological side effects.
commonly associated with NMDAR blockers.

6.1.1 Glycine and 6-hour time window

The FIRST OBJECTIVE of the present study is to test whether promoting neuronal survival by stimulating synaptic/NR2A NMDARs is an effective strategy for post-stroke treatment, compared to conventional strategy of blocking NMDARs. Once NMDAR’s downstream death signal becomes activated within 30 min to 1 h post-stroke onset, NMDAR blockers become ineffective against stroke damage. In CHAPTER 3 PART I, we demonstrate that stimulating NMDAR-mediated neuronal survival pathway with the NMDAR co-agonist glycine protects the rat brain against focal ischemic stroke even when the drug is administered 6 h post-ischemic onset. In fact, because neuronal death following stroke can take up to weeks to fully manifest (Dirnagl et al., 1999; Lipton, 1999), the effective therapeutic window of glycine, by stimulating NMDAR-based neuronal survival pathway, can be much longer than 6 h time-point tested in our study. Consistent with this possibility, we show that repeated daily doses of glycine is more neuroprotective against global ischemia than the single dose regimen, suggesting that glycine remains somewhat beneficial at least one day after ischemia.

6.1.2 Tat-INDIP and NMDAR-to-SREBP1 pathway

The SECOND OBJECTIVE of the present study is to test whether preventing neuronal death by inhibiting NMDAR-to-SREBP1 signaling may also offer a wider time window for drug administration. Among the NMDAR downstream death signals that are reported, we are particularly interested in SREPB1 because of its delayed time course - being activated only 6 h post-NMDA stimulation in vitro (Taghibiglou et al., 2009). In CHAPTER 3 PART II, we develop a therapeutic peptide Tat-INDIP that hinders SREBP1 activation by preventing
degradation of its partner protein insig1, and further demonstrate that this peptide protects the rat brain against focal ischemic stroke when administered 45 min pre- and 2 h post-stroke onset. In addition, because we are only blocking NMDAR-to-SREBP1 signaling, but not other NMDAR signaling pathways, our peptide is expected to convey much less side effects than conventional NMDAR blockers that act at the surface receptor level to block all NMDAR signaling pathways (Lai and Wang, 2010). Moreover, because SREBP1 remains strongly activated up to one week post-stroke, the therapeutic window of Tat-INDIP may be much wider than the 2 h tested in the present study.

6.1.3 Ubiquitin-tagged peptides and protein knockdown

The THIRD OBJECTIVE of the present study is to develop a novel method to directly knockdown proteins for degradation, as this would lead to therapeutics that can directly nullify NMDAR downstream-death signaling proteins. We report for the first time that ubiquitin-tagged biologics, in addition to their own degradation, can facilitate the degradation of non-tagged proteins via protein-protein interaction. This provides exciting possibility to knockdown any proteins through the protein-degradation pathways. This method can be used not only for biological and medical researches in the way DNA knockout mice and siRNA knockdown techniques are used (Castanotto and Rossi, 2009), but can also be clinically applicable in the way Tat-linked therapeutic peptides are used (Brooks et al., 2005; Vives et al., 1997). Moreover, this new method outperforms conventional techniques in that it allows differential knockdown of protein subtypes originating from the same pre-mRNA with alternative splicing (ie. Homer1a and Homer1b). Thus, this new method has research and clinical implications far beyond NMDAR-based stroke therapeutics.
6.2 Clinical Relevance and Potential Limitations

The central objective of the present study is to develop novel NMDAR-based therapeutics to protect neurons against excitotoxicity following stroke, with the ultimate goal of translating our findings to clinical use. As discussed in Chapter 1, our hypotheses were based on the understanding that conventional NMDAR antagonists failed clinical studies because of their short therapeutic time window; in contrast, novel NMDAR-based therapeutics introduced in this dissertation act by either stimulating the NMDAR survival-signaling pathway (Chapter 3), or by inhibiting a delayed-activated death-signaling protein downstream of the NMDAR death-signaling pathway (Chapters 4). Lastly, we introduced a new method to target other death-signaling proteins downstream of the NMDAR death-signaling pathway (Chapter 5). Our results (Chapter 3 and 4) and the results reported by others (discussed in Chapter 1: Aarts, et al., 2002; Liu, et al., 2007; Tu, et al., 2010; Zhang, et al., 2009; Zhou, et al., 2010) demonstrated that this novel NMDAR-based strategy for treating stroke indeed confer a much wider therapeutic time window compared to NMDAR antagonists.

Despite their advantages over conventional treatments, these therapeutic can confer their own clinical limitations. Nevertheless, the benefit of having wider therapeutic window can outweigh their potential limitations. The FIRST PART of this dissertation introduced the use of the NMDAR co-agonist glycine in stimulating neuronal survival (Chapter 3), and the SECOND and THIRD PART of this dissertation relied on the use of therapeutic peptides for stroke treatment (Chapters 3 and 4). Their direct clinical relevance and potential limitations are discussed as follows.
6.2.1 Potential limitations of glycine as a therapeutic agent

Although glycine demonstrated an impressive 4.5-6 h therapeutic time window against focal ischemic stroke, there is no experimental data suggesting that its side effects will be any more bearable than NMDAR blockers. That being said, we can predict its potential side effects based on findings on human patients suffering hyperglycemia. For instance, the most typical cases of severe chronic hyperglycemia result from a defect in the glycine cleavage enzyme, also known as the glycine synthase. Enzymatic degradation of glycine via this pathway is the predominant mechanism by which glycine is eliminated by the mammalian system, and human defect in this enzyme results in severe hyperglycemia characterized by mental deficiencies and early death.

Although these side effects may warn against chronic intake of glycine, it does not defeat the use of this therapeutic strategy for stroke treatment. First, there is no data suggesting that such chronic side effects will ensue with only a single dose of post-stroke administration. Second, pharmacokinetics and dose-response studies can be performed to outline the therapeutic-to-adversity ratio of glycine treatments, and such pharmacological measures may even allow safe chronic treatments. Third, in lieu of glycine, inhibitors of glycine transporter-1 (GlyT1) in the brain may be used to selectively elevate glycine concentration at the NMDAR-containing synapses (Depoortere et al., 2005), due to the selective presence of these transporters only at these synapses (Depoortere et al., 2005). Lastly, the present study provides the proof-of-concept that NMDAR-subtype-selective agonists for the NR2ARs can be designed and developed to become effective post-stroke therapeutics with wide therapeutic time window, and these may be further screened to identify the compound with minimal side effects.
6.2.2 Therapeutic peptides and clinical applicability

The successful identification of NMDAR downstream death-signaling pathways has made it possible to develop Tat-linked interference peptide to selectively inhibit the protein-protein interaction or the post-translational modifications required for NMDAR-mediated excitotoxic neuronal death following stroke (see Chapter 1). In this dissertation, we further introduced a novel method to knockdown targeted proteins for degradation via protein-protein interaction with an ubiquitin-tagged therapeutic peptide (Chapter 5). NMDAR-based therapeutics developed in this manner can specifically inhibit the death-signaling pathways, either through interference or knockdown, without affecting other functional-signaling or survival-signaling pathways. Thus, they do not have the side effects seen with traditional NMDAR blockers (Aarts et al., 2002; Lai et al., 2011; Zhou et al., 2010b). Moreover, because some of these pathways are activated long time after NMDAR activation, these therapeutics provide a much wider therapeutic time window - remaining effective long after NMDAR activation following stroke (Aarts et al., 2002; Liu et al., 2007; Tu et al., 2010; Zhang, 2009; Zhou et al., 2010b).

Although medical use of ‘peptidic biologics’ are generally limited by routes of drug administration due to their susceptibility to proteolytic enzymes, expensive cost for production, and risk for immunorejection (Lai and Wang, 2010), these limitations may not discourage the use of these interference peptides for stroke treatments. First, low bioavailability due to susceptibility to proteolytic enzymes can be minimized by intravenous injections in a hospital setting, where stroke patients are often treated. Secondly, the high cost and risk for immunorejection may not be a major issue as a single post-stroke dose is sufficient to achieve substantial neuroprotection (Aarts et al., 2002; Taghibiglou et al., 2009). Moreover, small molecules that mimic these interference peptides are already being developed through either high-throughput screening (Florio et al., 2009) or rationalized drug design.
(Zhou et al., 2010a). These small molecules retain most of advantages of the interference peptides but, are more clinically applicable than these peptides (Lai and Wang, 2010). Future studies should also investigate whether ubiquitin-tagged small molecules can also target proteins, via molecule-to-protein interaction, for degradation.

6.3 Future Directions

6.3.1 Development of NR2AR agonist

The present study provided the first proof-of-concept experimental results showing that synaptic/NR2ARs are pro-survival in the ischemic brain, and stimulating synaptic/NR2ARs is an effective strategy for protecting against stroke damage. In the absence of a selective NR2AR agonist, preferential stimulation of synaptic/NR2ARs was achieved using the NMDAR co-agonist glycine. Our rationale was based on the understanding that glycine only stimulates NMDARs in the co-presence of glutamate, which is normally only in the synapse (populated by NR2ARs). During stroke, however, glutamate is released to the extrasynaptic sites (populated by NR2BRs) due to reverse operation of the glutamate transporters (Colleoni et al., 2008; Kanthan et al., 1995; Rao et al., 2001; Rossi et al., 2000). Based on the “NMDAR location and subtype” hypotheses (Lai et al., 2011), glycine would normally be neuroprotective, but if given during stroke, would exacerbate stroke outcome. Indeed, blocking the glycine-binding site on NMDAR by administrating the drug before stroke onset improved stroke outcome in ischemic rats (Wood et al., 1992, 1993a). To prevent inadvertent stimulation of extrasynaptic/NR2BRs following stroke, we deliberately administered glycine at 3-6 h post-ischemia. In experimental stroke models, extrasynaptic glutamate release following stroke is short lived (Kanthan et al., 1995), which explains why glycine was neuroprotective when administered at this delayed time point.
The development of new compounds that selectively stimulates NR2ARs, either through high-throughput screening or *de novo* drug design, can allow safe drug administration at any time point before and after an ischemic episode. Moreover, such compound can be administered prior to predictable global ischemia, such as coronary-bypass surgeries (see Chapter 3 for discussion), without incidental stimulation of extrasynaptic/NR2BRs when glutamate release occurs.

### 6.3.2 Stroke intervention by targeting SREBP1 and beyond

As discussed in Chapter 4, SREBP1 is an important death-signaling protein downstream of the *NMDAR* excitotoxicity pathway. To interfere with NMDAR-to-SREBP1 signaling, a therapeutic peptide Tat-INDIP was developed to maintain the stability of INSIG1 following stroke. Based on the premises of this dissertation, once SREBP1 is activated, Tat-INDIP would no longer be effective against stroke damage. Therefore, a therapeutic that directly knockdown SREBP1 would confer wider therapeutic time window than would Tat-INDIP. Given that SREBP1 remains strongly activated up to one week following ischemic stroke (see Chapter 4), such therapeutic can have very impressive time window. Notably, we have developed a method to directly knockdown proteins via degradation pathways; specifically, we found that ubiquitin-tagged biologics can knockdown a target protein, via direct protein-protein interaction, through proteasomal degradation (see Chapter 5). The protein-protein interaction site of the ubiquitin-tagged biologic for targeting SREBP1 can be commercially identified through factory-scale yeast two-hybrid libraries, and can even be designed to be selective against the active form of SREBP1 (or the nt-SREBP1) but not the native form. Permeability across the cell membrane and the BBB can be achieved by Tat-fusion or polyarginine fusion (see Chapter 1.6.3 for detailed discussion).
Aside from SREBP1, the gene-products downstream of SREBP1 can also be potential targets for developing stroke treatments. As a transcription factor, SREBP1 can promote neuronal death by downregulating survival-promoting genes, upregulating death-promoting genes, or both. The transcriptome of NMDAR-induced SREBP1 activity has not been mapped out. Future studies can investigate this by means of real time PCR; based on the notion that targeting downstream signals can confer wider therapeutic window, such finding can lead to the development of stroke therapeutics with even wider window than targeting SREBP1 directly.

6.4 Concluding Remarks

The results presented in this doctoral dissertation represent some of the major milestones in the continuous endeavor to develop novel NMDAR-based therapeutics in the stroke research field. The primary goal in translational stroke research has been to develop therapeutics that provide a wide time window, and with minimal side effects. We report here that glycine, by stimulating NMDAR-mediated survival pathway, is strongly neuroprotective even when administered 4.5-6 h post-ischemia (Part I). Moreover, we identified SREPB1 as a death signaling protein downstream of NMDARs, and developed a therapeutic peptide Tat-INDIP to inhibit NMDAR-to-SREBP1 signaling (Part II). Because Tat-INDIP targets a single NMDAR signaling pathway, rather than all NMDAR signaling pathways, it is expected to have much fewer side effects than conventional NMDAR blockers. Finally, in light of the recent discovery of many NMDAR downstream death-signaling proteins, we invented a general method for directly targeting proteins of interest, via protein-protein interaction with an ubiquitin-tagged biologic, for proteasomal degradation (Part III). This new method not only hold promises for future development of NMDAR-based stroke treatment, but can have great
impact in biological/medical research and clinical treatment of other diseases.

Notably, because NMDAR-mediated excitotoxicity is thought to be a common neuropathology associated with a large number of neurological disorders ranging from acute brain insults such as brain trauma to chronic neurodegenerative disorders such as Huntington’s Disease and Parkinson’s Disease (Lipton and Rosenberg, 1994), NMDAR-based therapeutics described in this doctoral dissertation can have broad implications beyond stroke, raising the exciting potential for designing new therapeutics for the clinical treatment of these neurological disorders.
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