OPPOSING ROLES OF NMDA RECEPTOR SUBTYPES IN NEURONAL FATE AND NOVEL TREATMENTS FOR ISCHEMIC BRAIN INJURY

by

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ABSTRACT

Ischemic brain damage is largely due to excitotoxicity mediated by glutamate receptors, notably the N-methyl-D-aspartate-type receptors (NMDARs); however, to date none of the NMDAR antagonists have shown therapeutic benefits in treating stroke. Moreover, recent studies indicate blockade of NMDARs may even cause neuronal death. An explanation of the molecular mechanisms underlying this paradox is urgently required in order to develop new, effective stroke therapeutics. In this doctoral thesis project, we hypothesized that different NMDAR subtypes have opposing roles in neuronal fate and effective treatment of ischemic brain injury may be achieved through selective activation of the NMDAR subtype mediating neuronal survival and/or blockage of the NMDAR subtype mediating neuronal death.

We first determined whether the two major NMDAR subtypes in mature cortical neurons, NR2A- and NR2B-containing NMDARs play differential roles in neuronal apoptosis. The results showed that NR2B-containing NMDARs are coupled to neuronal death whereas NR2A-containing receptors mediate neuronal survival. Further investigation revealed that the subcellular location (synaptic versus extrasynaptic) of NMDARs has little effect on their roles in neuronal fate. We then tested whether selective activation of NR2A-mediated neuronal survival signaling or inhibition of NR2B-mediated neuronal death pathway is neuroprotective in stroke models. The data showed that blockade of NR2B is neuroprotective but has a relatively narrow therapeutic window. In contrast, selective activation of NR2A attenuates ischemic brain injury even when delivered 4.5 h post stoke onset. These findings suggest for the first time that selective stimulation of NR2A-containing NMDARs may constitute a promising therapy for stroke. Due to the critical role of NR2B in neuronal death

and the narrow time window of NR2B antagonists, we examined whether disrupting the excitotoxic signaling pathway downstream of NR2B activation was efficacious in treating ischemic damage. We found that post-ischemic administration of an interference peptide derived from the carboxyl tail of NR2B remarkably reduces stroke-induced brain injury. Thus, perturbing protein-protein interaction downstream of NR2B activation may represent another novel therapy for stroke.

This research project provides a molecular basis for the dual roles of NMDARs in neuronal survival and demise and thereby suggests a number of clinically relevant new stroke therapies.

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Abbreviation	Definition
ACSF	Artificial cerebrospinal fluid
AIF	Apoptosis-inducing factor
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid
AMPAR	α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid type glutamate receptor
ANOVA	Analysis of variance
AP-5/APV	2-amino-5-phosphonopentanoic acid
Apaf-1	Apoptosis protease-activating factor 1
ASIC	Acid-sensing ion channel
Bcl-2	B-cell lymphoma-2
CaMKII	Calcium-calmodulin kinase II
CARD	Caspase recruitment domain
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
Co-IP	Coimmunoprecipitation
Cyt C	Cytochrome C
DED	Death effector domain
DIABLO	Direct IAP binding protein with low pI
DISC	Death-inducing signaling complex
DIV	(cultures) Day in vitro
DNQX	6,7-dinitroquinoxaline-2,3-dione
ECS	Extracellular solution
Endo G	Endonuclease G
EPSC	Excitatory postsynaptic current
ER	Endoplasmic reticulum
Erk	Extracellular signal-regulated kinase
FADD	Fas-associated adaptor protein with death domain
fEPSC	Field excitatory postsynaptic current
GABA	γ-aminobutyric acid
H&E	Hematoxylin and eosin staining
ICA	Internal cerebral artery

iGluR	Ionotropic glutamate receptor
KA	Kainic acid
MCA	Middle cerebral artery
MCAo	Middle cerebral artery occlusion
mEPSC	Miniature excitatory postsynaptic current
MOMP	Mitochondrial outer membrane permeabilization
mRFP	Mutant red fluorescence protein
NCX	Na+/Ca2+ exchanger
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate-type glutamate receptors
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
OGD	Oxygen-glucose deprivation
PDZ	PSD-95, Dlg, and ZO-1 homology domain
PI3K	Phosphatidylinositol 3-kinase
PSD	Postsynaptic density
PT	Permeability transition
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
RasGRF	Ras guanine nucleotide-releasing factor
RNAi	RNA interference
ROS	Reactive oxygen species
SAP	Synapse associated protein
SEM	Standard error of mean
siRNA	Small interfering RNA
Smac	second mitochondria-derived activator of caspase
SynGAP	Synaptic GTPase activating protein
tBid	Truncated Bid
VGCC	Voltage-gated calcium channel
XOD	Xanthine oxidase

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"If I have seen further it is by standing on ye shoulders of Giants." -Isaac Newton

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CHAPTER 1: INTRODUCTION

1.1. Overview

Stroke is now a major cause of morbidity and mortality in most industrialized countries (Hacke et al., 1991;Dirnagl et al., 1999). In the United States alone, every 45 seconds there is a person suffering a stroke and every 3.1 minutes someone dies of a stroke (American Heart Association, 2004) . In addition to its devastating effect on the patient and his/her family, it also imposes an enormous burden on a nation's economy (Ter Horst and Postigo, 1997;Lin, 2002). According to the estimates, the annual cost of stroke-related expenses in the US accumulates up to approximately 71.8 billion dollars (Hinkle and Bowman, 2003;Murphy, 2003). These statistics suggest that a cure for stroke is urgently needed.

Current therapies for ischemic stroke can be classified into two categories: neuroprotective therapy and thrombolytic therapy (Fisher and Bogousslavsky, 1998). The rationale of neuroprotective therapy lies in that brain ischemia triggers a series of pathological changes that result in neuronal death. Theoretically, it is possible to salvage the brain tissue if the neurotoxic events can be promptly interrupted and/or an inherent neuroprotective ability can be activated in time. In accord, many neuroprotectants have been developed in hope of diminishing brain damage resulting from stroke (Lutsep and Clark, 2001;Hinkle and Bowman, 2003;Danton and Dietrich, 2004;Wahlgren and Ahmed, 2004;Beresford et al., 2003); however, none of these agents, including many glutamate receptor antagonists, have exhibited clinical efficacy. Thrombolytic therapy takes advantage of the fact that clinical ischemic

stroke is mostly due to disruption of cerebral blood flow by thrombosis. Therefore, thrombolysis may reconstitute the circulation of the ischemic brain regions and hence prevent further brain injury (Jenkinson, 2004;Cornu et al., 2001;del Zoppo, 2004). To date the thrombolytics that have been investigated include recombinant tissue-type plasminogen activator (rt-PA), streptokinase, urokinase and prourokinase (Cornu et al., 2001;Madden, 2002;Hawn and Baldwin, 1997). rt-PA is currently the only drug approved by FDA for the treatment of acute stroke; however, whether it is effective remains controversial (Hacke et al., 1998;Albers et al., 2002). Furthermore, rt-PA has a narrow therapeutic time window (Mielke et al., 2004;Schellinger and Warach, 2004;Tomsick, 2004;Madden, 2002), which allows no more than 7% of stroke patients to receive this therapy (Cocho et al., 2005). In other words, thrombolytic therapy for stroke is also far from satisfactory (Caplan, 2004).

Given the clear role of glutamate receptors, especially the NMDA subtype in excitotoxicity (Lipton and Rosenberg, 1994), it is paradoxical that none of the NMDAR antagonists have succeeded in stroke clinical trials to date. Further understanding of the functioning of NMDARs during ischemic stroke is critical. It has been known that NMDARs have several subtypes depending on their subunit composition, and further investigation have shown that these subtypes possess distinct pharmacological and electrophysiological properties; however, the functional differentiation of these NMDAR subtypes are not well-studied. Most recently, it has been found that the two major NMDAR subpopulations, NR2A- and NR2B-containing NMDARs, may govern the polarity of synaptic plasticity in the cortex and hippocampus (Liu et al., 2004a;Massey et al., 2004b), indicating NMDAR subtypes

may be functionally diverse.

In this thesis project, I hypothesized that the roles of NMDARs in excitotoxicity are also dependent on the NMDAR subtype activated. I first studied the effects of activation of the two major NMDAR subtypes in mature cortical neurons, NR2A- and NR2B-containing NMDARs, on neuronal apoptosis. The results showed that NR2A-containing NMDARs have anti-apoptotic activity while their NR2B-containing counterparts mainly mediate neuronal death. Based on these discoveries, two new therapies for stroke were developed: the first was to specifically activate NR2A-containing NMDARs, and the second was to disrupt the neurotoxic signaling pathway downstream of activation of NR2B-containing NMDARs (in collaboration with Dr. Michael Tymianski's lab). These two strategies were shown to have dramatic effects in attenuating ischemic brain damage *in vivo* either prior to or, most importantly, following stroke onset. These findings suggest specifically enhancing the survival-promoting action of NR2A-containing NMDARs and/or perturbing the signaling pathway downstream of activation of the apoptosis-mediating NR2B-containing NMDARs may avoid the drawbacks of current NMDAR antagonists in treating stroke and constitute promising therapies for stroke.

1.2. Ischemic stroke and stroke models

Being one of the most active organs in the body, the brain has a huge energy demand. Under physiological conditions, the majority of energy is produced through oxidative phosphorylation of glucose, which requires not only glucose as a substrate but also an adequate supply of oxygen (O_2). It is estimated that although it only represents 2% of

the total body weight, the brain consumes about 25% of the total body glucose utilization and 20% of the resting total body O_2 (Clarke and Sokoloff L., 1994;Brust, 2000); however, the O_2 storage in the brain is miniscule. Thus, the brain is dependent upon continuous replenishment of O_2 through cerebral circulation (Clarke and Sokoloff L., 1994).

Ischemic stroke is a condition in which the cerebral blood flow is abruptly cut off or reduced drastically, resulting in lack of O_2 and glucose in the ischemic regions. Naturally-occurring cerebral ischemia falls into two types: focal brain ischemia and global ischemia (Neumar, 2000). Focal ischemia is more commonly seen in humans. It may happen when a cerebral artery, such as the lenticulostriate artery or middle cerebral artery (MCA), is blocked by thrombosis or embolism. In contrast, global ischemia is usually caused by a systematic reduction in blood flow like a cardiac arrest.

As aforementioned, stroke has a tremendous negative impact on the society but no effective cure for stroke has been discovered. To study the mechanisms of ischemic brain injury, many experimental stroke models have been developed over the past years. At present, two types of stroke models, i.e. *in vitro* and *in vivo* models, have been established. *In vitro* experimental strokes can be produced in either brain slices or cell cultures. One most widely used paradigm *in vitro* is the oxygen-glucose deprivation (OGD) model. The bathing solution for brain slices or cultured cells is rapidly changed from O_2/CO_2- to N_2/CO_2-equilibrated solution without glucose. The lack of blood flow in stroke models *in vitro* makes it easier to interpret the obtained results; however, cerebral blood flow is an important factor that affects the outcome

of stroke. In addition, the anatomical relationship between an *in vivo* brain and *in vitro* cultured cells is not the same, and hence the changes observed *in vitro* may not reflect the situations *in vivo* (Lipton, 1999a).

Animal models have long been used to study stroke. In correspondence with the cerebral ischemia that occurs in humans, strokes models *in vivo* are divided into two types: global and focal ischemia (Ginsberg and Busto, 1989). Global ischemia is produced by occlusion of vessels that results in an ischemia affecting a large portion of the forebrain. Global ischemia usually leads to selective cell death in the CA1 region of the hippocampus. Delayed cell death is a major feature of global ischemia (Pulsinelli and Brierley, 1979;Kirino, 1982;Pulsinelli et al., 1982). The more frequently used model *in vivo* is the focal ischemic model, which usually involves occlusion of the MCA (MCAo) (Longa et al., 1989;Ginsberg and Busto, 1989;Strong et al., 1983;Symon, 1975). Among the different techniques of MCA occlusion, one well-established and widely used method is to insert a nylon suture through internal carotid artery (ICA) until the tip of the suture reaches the point at which the MCA branches from ICA so that MCA is blocked at its origin. In this model, there is a gradient in the ischemic region. In the core area, the blood flow is reduced to less than 15% of normal value (Duverger and MacKenzie, 1988;Nedergaard et al., 1986;Sakatani et al., 1990;Tamura et al., 1981), while in the region surrounding the core, which is termed penumbra, the blood flow is about 15-40% of normal (Ginsberg and Busto, 1989;Hossmann, 1994;Back et al., 1995). There is also the peri-infarct region whereby the blood flow is reduced but remains above 40% of normal. Focal ischemia produces a contiguous mass of damaged brain tissue termed infarct instead

of selective lesion in certain vulnerable regions of the brain.

1.3. Neuronal death following ischemic stroke

Post-ischemic neurons mainly exhibit characteristics of two distinct forms of cell death: necrosis and apoptosis, depending on the intensity of the insults (Chopp and Li, 1996;Majno and Joris, 1995;Snider et al., 1999;Yuan et al., 2003;Pettmann and Henderson, 1998). Necrosis progresses rapidly and it is morphologically characterized by cellular and organelle swelling followed by disruption of nuclear, organelle and plasma membranes, along with disintegration of nuclear structure and cytoplasmic organelles (Majno and Joris, 1995;Clarke, 1990). Later in necrosis, chromatin may disappear entirely. Cells are killed by lipolysis, proteolysis and loss of ion homeostasis (Dirnagl et al., 1999;Aarts et al., 2003b). Inflammation may ensue because of the release of cellular contents into the extracellular space (Dirnagl et al., 1999;Neumar, 2000). In contrast, apoptosis is a form of delayed and programmed cell death (Yuan and Yankner, 2000). Apoptotic cells display characteristics of compaction and margination of the nuclear chromatin, cytoplasmic shrinkage and condensation with preservation of organelles, and nuclear and cytoplasmic budding to form membrane-bound fragments, i.e. apoptotic bodies (Kerr et al., 1972;Yuan et al., 2003;Neumar, 2000). Under appropriate conditions, apoptotic bodies are engulfed by macrophages and hence cellular contents are usually not spilled into extracellular space. At the molecular levels, apoptosis can be distinguished by exposure of phosphatidylserine (PS) on the outer surface of the plasma membrane, cleavage and activation of caspases, and eventual DNA fragmentation (Danial and Korsmeyer,

2004;Yuan and Yankner, 2000;Thornberry and Lazebnik, 1998).

Until the early 1990s, ischemic brain damage was generally thought to result exclusively from necrosis, but in recent years mounting evidences have shown that apoptosis is an important part of ischemic brain injury (Sims and Anderson, 2002;Chopp and Li, 1996;Choi, 1996;Love, 2003;Love, 2003). Whether ischemic neurons die through necrosis or apoptosis depends heavily on the extent and duration of ischemia (Lipton, 1999a). Necrosis occurs within the ischemic core, whereas apoptosis is typically seen in the penumbra region (Juurlink and Sweeney, 1997). In the ischemic core of a transient focal ischemia, the ratio of necrotic to apoptotic cells is estimated to be around 1:1, whereas in the penumbra, the ratio is about 1:9 (Charriaut-Marlangue, 2004). Given these mechanisms and the rapidity with which the process occurs, necrosis is usually regarded as an uncontrolled/unregulated process. On the contrary, apoptosis has been shown to be tightly controlled, and the signaling pathways leading to apoptosis have been delineated over the past years.

1.4. Mechanisms of ischemic neuronal death

The precise mechanisms of post-ischemic neuronal death are yet to be determined albeit the extensive studies conducted. Advances in this research area over the past years, however, have greatly improved our understanding of how ischemic brain injury occurs. It is now believed that ischemic neuronal death may occur through an interplay between alteration of Ca^{2+} homeostasis, reduced ATP production, elevated generation of reactive oxygen species (ROS) and acidosis.

Ca^{2+} is a vital intracellular messenger governing cellular functions, such as synaptic

activity and membrane excitability. Neurons maintain a tight command of Ca^{2+} homeostasis, including both intracellular levels and distribution of Ca^{2+}, through an interplay between Ca^{2+} influx and efflux, Ca^{2+} buffering and internal Ca^{2+} storage (Arundine and Tymianski, 2003). At resting state, the concentration of free Ca^{2+} in the cytosol is kept at low levels (~100 nM). During stroke, intracellular Ca^{2+} concentration increases dramatically. Ca^{2+} influx through glutamate receptors, especially the NMDA subtype, has been found to be a major source of the intracellular Ca^{2+} increase (Arundine and Tymianski, 2004a;Choi, 1985;Choi, 1995). Ca^{2+} released from intracellular Ca^{2+} stores such as endoplasmic reticulum (ER) and mitochondria may contribute to the disruption of Ca^{2+} homeostasis as well (Hayashi and Abe, 2004;Ganitkevich, 2003;Paschen, 2000;Paschen and Doutheil, 1999). Most recently, it is shown that the major plasma membrane Ca^{2+} extruding system, the Na^{+}/Ca^{2+} exchanger (NCX), also plays a critical role in delayed Ca^{2+} elevation in neurons (Bano et al., 2005). NCX3 is cleaved in neurons undergoing excitotoxicity, and this cleavage aggravates Ca^{2+} accumulation in neurons. Although voltage-gated Ca^{2+} channels (VGCCs) are an important site of calcium entry, it is still controversial whether this Ca^{2+} influx is toxic (Hardingham et al., 2002;Bading et al., 1993;Hardingham et al., 1999). Thus, the "source specificity" hypothesis proposes that Ca^{2+} toxicity occurs through Ca^{2+}-signaling pathways linked to specific routes of Ca^{2+} influx (Sattler and Tymianski, 2000;Tymianski et al., 1993). Whatever the source is, *in vivo* studies have demonstrated cytosolic and mitochondrial Ca^{2+} levels are dramatically elevated during ischemia and early reperfusion. A brief global ischemia can trigger a ~2,000-fold elevation in the selectively vulnerable

hippocampal CA1 and cortical neurons (Silver and Erecinska, 1992;Erecinska and Silver, 1992). In focal ischemia, total tissue Ca^{2+} in both ischemic core and penumbra increases and lasts up to 24 hours after reperfusion (Kristian et al., 1998). Mitochondrial Ca^{2+} elevation in the ischemic core is proportional to the duration of ischemia (Gutierrez-Diaz et al., 1985). Ca^{2+} disregulation is paramount to neuronal death (Arundine and Tymianski, 2003;Kristian and Siesjo, 1998). Intracellular Ca^{2+} overload can trigger activation of lipases, proteases, endonucleases and kinases/phosphatases which eventually leads to neuronal death; however, the exact mechanism by which Ca^{2+} mediates excitotoxicity is still unclear.

Under physiological conditions, mitochondria are capable of sequestering large amounts of intracellular Ca^{2+}; however, abnormal accumulation of Ca^{2+} during stroke can cause mitochondrial dysfunction. Ca^{2+} is sequestered into the mitochondrial matrix via a proton electrochemical gradient that is generated by the electron transport chain and depolarizes the mitochondrial potential (Akerman, 1978;Gunter and Pfeiffer, 1990;Loew et al., 1994). This influx of Ca^{2+} results in a reduction in the electrochemical gradient, and consequently the reduction of ATP production. In the meantime, more ATP is consumed by cells in order to extrude the intracellular Ca^{2+} overload. It's estimated that a 10-minute global ischemia can lead to a drop of ATP levels to 10% or less of normal values (Wagner and Lanier, 1994). In focal ischemia, the loss of ATP is less drastic, with ~25% and ~50-70% of normal in the ischemic core and penumbra, respectively (Sun et al., 1995;Welsh et al., 1991). The concurrent accumulation of intramitochondrial Ca^{2+}, decreased ATP generation and increased ATP consumption are critical in mediating early ischemic cell death (Schinder et al.,

1996).

ROS, including oxygen free radicals, nitric oxide (NO) and peroxynitrite ($ONOO^-$), the reaction product of superoxide and NO, are important mediators of ischemic neuronal death. Neurons are exposed to a baseline of free radical-mediated oxidative stress that is presumably tolerated by the cells. When ROS production exceeds the normal neuronal buffering capacity, it can impinge on neuronal integrity. Impairment of mitochondria during ischemia results in deficits in mitochondrial electron transport chain, which in turn leads to excessive free radical production (Green and Reed, 1998;Antonsson et al., 1997;Kumar et al., 1990). Free radicals can be generated through several pathways. For example, the breakdown of adenine nucleotides leads to accumulation of hypoxanthine. In the meantime, xanthine oxidase (XOD) production is increased as well (Mishra and ivoria-Papadopoulos, 1999). XOD can metabolize hypoxanthine to produce free radicals (Sussman and Bulkley, 1990). In addition, arachadonic acid accumulates during cerebral ischemia (Zhang and Sun, 1995), and arachadonic acid metabolism by oxidases can generate free radicals as well. Studies *in vivo* have demonstrated that the production of free radicals is elevated during and after global and focal ischemia (Kumar et al., 1990). In neurons, NO is mainly synthesized from L-arginine through the catalytic activity of neuronal NO synthase (nNOS). Ischemia dramatically increases nNOS activation in a calcium/calmodulin-dependent manner (Zhang et al., 1994;Dawson et al., 1996). More recently, it is shown that NMDAR activation is specifically linked to NO production via the post synaptic density (PSD) scaffolding protein PSD-95 (Sattler et al., 1999). NO production rises to the low micromolar range during global and focal

ischemia and elevated after focal ischemia as well (Lipton, 1999a). NO has many roles in the central nervous system (CNS) as a messenger molecule; however, it can be neurotoxic when generated in excess (Dawson and Dawson, 1996;Lipton, 1999b). On the injurious side, NO can damage DNA, eventually leading to neuronal death (Li and Wogan, 2005;Martin et al., 2005). NO also inhibits mitochondrial respiratory chain enzymes (Murray et al., 2003), and reacts with superoxide anion to produce $ONOO^-$. Although there is no direct evidence showing that $ONOO^-$ production is elevated, the increased generation of superoxide and NO should allow formation of $ONOO^-$ (Beckman, 1994). $ONOO^-$ is a potent oxidant that reacts with sulphydryls and with zinc-thiolate moieties. It can also react with nitrate and hydroxylate aromatic rings on amino acid residues to oxidize lipids, protein and DNA (Aarts et al., 2003b). An array of studies over the past years has shown that ROS play a central role in the development of ischemia-induced neuronal damage (Chan, 2001;Kontos, 2001).

Complete oxidation of glucose is required for the brain to fulfill its energy requirement. A lack of blood supply and hence a shortage of oxygen following ischemia inevitably perturbs oxidative phosphorylation and forces neurons to switch to anaerobic glycolysis. Consequently, excessive lactic acid is produced as a byproduct of glycolysis, and protons also accumulate due to ATP hydrolysis. These all contribute to the drop of pH value in the ischemic tissue (Siesjo et al., 1996;Rehncrona, 1985). Under normoglycemic conditions, tissue pH can fall to as low as 6.5-6.0 during ischemia; however, if a hyperglycemia exists or an ischemia is extremely severe, pH can drop even lower (below 6.0) (Siesjo et al., 1996;Rehncrona, 1985;Nedergaard et al., 1991a;Nedergaard et al., 1991b). Acidosis has been shown to

greatly exacerbate ischemic brain damage (Huang and McNamara, 2004;Siesjo et al., 1996;Tombaugh and Sapolsky, 1993). Most recently, a novel mechanism by which acidosis induces neuronal death has been revealed (Yermolaieva et al., 2004;Xiong et al., 2004). It has been shown that acidosis activates acid-sensing ion channels (ASICs) and hence elicits Ca^{2+} influx independent of glutamate receptors. Furthermore, both ASIC blockers and knockout of ASIC gene can protects the brain from ischemic injury.

1.5. Signaling pathways leading to neuronal apoptosis

As discussed, neuronal apoptosis is a tightly-controlled cell death pathway that develops relatively slowly when compared to necrosis. Therefore, many neuroprotective approaches target different intermediate steps leading to neuronal apoptosis following stroke. Two apoptotic pathways have been characterized so far: the intrinsic and extrinsic apoptotic pathways.

In post-ischemic neurons, neurotoxic events such as ATP depletion, intracellular Ca^{2+} overload and generation of ROS may converge on mitochondria to induce mitochondrial outer membrane permeabilization (MOMP), and hence cytochrome C (Cyt C) release (Aarts and Tymianski, 2004;Zipfel et al., 2000;Neumar, 2000;Nicholls, 2004), which initiates intrinsic apoptotic pathway. Therefore, mitochondria are generally thought to play a central role in this signaling pathway (Zamzami and Kroemer, 2001;Yuan and Yankner, 2000;Green and Reed, 1998;Finkel, 2001;Cai et al., 1998;Mignotte and Vayssiere, 1998). The mechanisms responsible for MOMP during apoptosis are still at issue (Halestrap et al., 2002a). It is argued that

MOMP is closely related to the B-cell lymphoma-2 (Bcl-2) family proteins (Sharpe et al., 2004;Kuwana and Newmeyer, 2003;Scorrano and Korsmeyer, 2003;Wei et al., 2001;Letai et al., 2002). The Bcl-2 family of apoptosis-regulating proteins contains both anti- and pro-apoptotic members which reside immediately upstream of mitochondria (Chan and Yu, 2004;Harada and Grant, 2003;Tsujimoto and Shimizu, 2000;Tsujimoto, 1998). Anti-apoptotic Bcl-2 proteins such as Bcl-2 and Bcl-XL function to block MOMP while the pro-apoptotic members promote it (Halestrap et al., 2002b;Wei et al., 2001;Letai et al., 2002;Degli and Dive, 2003). Another mechanism of MOMP may be the opening of the permeability transition (PT) pore, which results in loss of mitochondrial inner transmembrane potential ($\Delta\Psi m$) and swelling of the matrix. After CytC is released from mitochondria, it binds to apoptosis protease-activating factor 1 (Apaf-1) (Hill et al., 2003). Apaf-1 is a cytosolic protein that contains a caspase recruitment domain (CARD), a nucleotide-binding domain and several WD-40 domains (Lauber et al., 2001;Zou et al., 1999). Following the binding of Apaf-1 to CytC, the nucleotide dATP or ATP binds to the complex and triggers its oligomerization to form an apoptosome (Hill et al., 2003). The CARD domain is then exposed in the apoptosome, which subsequently recruits procaspase 9. Recruitment of procaspase 9 leads to its autoactivation through cleavage into caspase 9. The active caspase 9 then cleaves procaspase 3 into caspase 3, the active form of the enzyme (Porter and Janicke, 1999). Caspase 3, along with other effector caspases such as caspase 6 and 7, is the major effector caspase in apoptosis (Slee et al., 2001;Van de et al., 1999;Stennicke and Salvesen, 1997). Caspases are aspartate-specific cysteine proteases which can cleave proteins such as

the DNA-repairing enzyme poly (ADP-ribose) polymerase (PARP) and the cytoskeleton protein, gelsolin, and result in neuronal disassembly (Love, 2003). MOMP also results in the release of other intermitochondrial membrane proteins such as second mitochondria-derived activator of caspase (Smac)/direct IAP binding protein with low pI (DIABLO), apoptosis-inducing factor (AIF) and endonuclease G (Endo G) (Du et al., 2000;Verhagen et al., 2000;Lu et al., 2003;Cande et al., 2002;Widlak and Garrard, 2005;Schafer et al., 2004;Arnoult et al., 2003;Li et al., 2001;Li et al., 2001). Smac/DIABLO can promote CytC/Apaf-1-dependent caspase activation. AIF and Endo G, on the other hand, can mediate caspase-independent forms of apoptosis. Neuronal death that follows experimental brain injury has been shown to involve AIF translocation from the mitochondria to cell nuclei (Zhang et al., 2002). Endo G is a DNase that induces nuclear DNA cleavage and apoptosis, which may be translocated from mitochondria to nucleus following cerebral ischemia (Li et al., 2001;Lee et al., 2005).

Death receptor-dependent extrinsic apoptotic pathway may be involved in ischemic neuronal death as well (Love, 2003;Felderhoff-Mueser et al., 2000;Carboni et al., 2005). Cerebral ischemia may upregulate death receptors such as Fas/CD95 (Felderhoff-Mueser et al., 2000;Carboni et al., 2005). The mechanism is still not fully understood but p53 levels can be increased by ischemic injury, and p53 is capable of upregulating Fas/CD95 (Rich et al., 2000). Upon binding of Fas ligand (FasL), Fas receptors trimerize and undergo a conformational change, which subsequently assembles on the cytoplasmic tail a signaling complex known as the death-inducing signaling complex (DISC) (Muzio et al., 1996). The Fas-associated adaptor protein

with death domain (FADD) binds to Fas via its DD domain and recruits procaspase 8 via its death effector domain (DED) (Kischkel et al., 1995). Procaspase 8 is then activated through autoproteolysis. Following activation of caspase 8, there are two possible pathways leading to apoptosis (Scaffidi et al., 1998). The first is through cleavage of caspases 3 and 7, and the other pathway involves truncation of Bid. Truncated Bid (tBid) can be translocated to mitochondria and hence trigger the intrinsic apoptotic pathway (Li et al., 1998a;Luo et al., 1998).

1.6. NMDARs as therapeutic targets for stroke

Glutamate is a major neurotransmitter in the mammalian central nervous system (CNS). Glutamate receptors are divided into two pharmacologically and functionally distinct families, i.e. metabotropic and ionotropic glutamate receptors (mGluRs and iGluRs). Given the main research aims in this project, here I will only focus on one type of iGluRs, i.e. NMDARs.

1.6.1. NMDARs

Based on their affinity to three selective agonists, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA) and kainic acid (KA), iGluRs can be divided into three subfamilies, i.e. NMDARs, AMPA receptors (AMPARs) and kainate receptors, respectively (Erreger et al., 2004;Dingledine et al., 1999). Each subfamily is further divided into distinct subunits that come together to form a homo- or heteromeric ion channels (Table 1).

Table 1. Ionotropic glutamate receptor subunits

Receptor family	Subunit	Selective agonist
NMDARs	NR1	NMDA
	NR2A, NR2B, NR2C, NR2D	
	NR3A, NR3B	
AMPARs	GluR1	AMPA
	GluR2	
	GluR3	
	GluR4	
Kainate receptors	GluR5	Kainic acid
	GluR6	
	GluR7	
	KA1	
	KA2	

NMDARs were first cloned around 1990. Three types of NMDAR subunits, NR1, NR2 and NR3, are encoded by three distinct gene families (Dingledine et al., 1999). Transmembrane topology study indicates that each subunit has four hydrophobic membrane-spanning domains, three of which are transmembrane domains (M1, M3 and M4) and one is a cytoplasmic-facing re-entrant membrane loop (M2) (Dingledine et al., 1999;Kuner et al., 1996). The N terminus of each subunit is located extracellularly and the C terminus intracellularly. The M2 domain lines the pore of the ion channel (Kuner et al., 1996). In the homologous regions of the NR2 subunit resides the agonist (glutamate or NMDA) binding site. Interestingly, activation of NMDARs requires a co-agonist, glycine or D-serine (Kleckner and Dingledine, 1988;Fern et al., 1996;Mayer et al., 1989). The co-agonist binding site is formed by the region preceding segment M1 (S1 domain) and the loop region between M3 and M4 (S2 domain) of the NR1 subunit (Laube et al., 1998;Kuryatov et al., 1994;Dingledine et al., 1999).

Using a model based on random aggregation of NMDAR subunits, it was first proposed that NMDARs be a pentameric structure (Premkumar and Auerbach, 1997). But later studies favored an assembly of four subunits (Rosenmund et al., 1998;Laube et al., 1998). Functional analyses *in vitro* have shown that only the recombinant heteromers, but not the homomer of either NR1 or NR2A-D, match the physiological and pharmacological responses of the native NMDARs (Janssen et al., 2005). Therefore, it is now generally believed that NMDARs are heterotetramers comprised of two copies of the NR1 subunit and two copies of NR2A-D subunit (complexes formed by NR1 and NR3A or 3B have been shown to be gated by glycine (Chatterton

et al., 2002) and hence not classical NMDARs). Some native NMDARs may be triheteromers which contain NR1 and two different types of NR2 subunit (Cull-Candy et al., 2001;Sheng et al., 1994), e.g., NR1/NR2A/NR2B; however, the ratio of triheteromeric vs. diheteromeric NMDARs in the brain is still under debate (Balhos and Wenthold, 1996; Luo et al., 1997).

The distribution of NMDAR subtypes in the mammalian nervous system undergoes a developmental change (Monyer et al., 1994;Akazawa et al., 1994;Sheng et al., 1994;Li et al., 1998b). NR1 subunit is ubiquitously expressed throughout development and adulthood. NR2A expression cannot be detected until soon after birth, but it increases gradually when approaching adulthood. Conversely, the levels of NR2B subunit are high during the prenatal period but gradually decrease with development. The expression NR2C first appears at the third postnatal week and increases steadily. Similar to NR2B, NR2D is highly expressed prenatally, but the levels of NR2D drop quickly after birth. In an adult CNS, NMDAR subtypes distribute differentially (Monyer et al., 1994). The NR2A-containing NMDARs are widely distributed, with high levels in the forebrain, hippocampus, and cerebellum. NR2B-containing NMDARs are highly represented in the forebrain, striatum and midbrain. The adult cerebellum has the greatest concentration of the NR2C-containing receptors, while NR2D-containing NMDARs is weakly expressed in the brainstem and spinal cord. The distribution of NMDAR subtypes within a neuron may differ as well. NMDARs are mostly localized to the postsynaptic sites. It has been shown in cortical and hippocampal neurons that NR2A-containing NMDARs are preferentially located at excitatory synapses, whereas NR2B-containing

NMDARs are predominant at extrasynaptic sites (Tovar and Westbrook, 1999;Stocca and Vicini, 1998). NR2D-containing NMDARs are present extrasynaptically in dorsal horn spinal neurons (Momiyama, 2000) but there is no evidence for the presence of synaptic NR1/NR2D receptors (Cull-Candy et al., 2001). In contrast, NR1/NR2C receptors can be found at synapses in cerebellum (Cathala et al., 2000).

Compared with AMPAR and kainate receptors, NMDARs activate slowly and deactivate with a much slower time course. In the continued presence of glutamate, the responses of NMDARs to the agonist are diminished, i.e., NMDARs desensitize. Three forms of desensitization of NMDAR channels have been identified: glycine-dependent desensitization, glycine-independent desensitization and Ca^{2+}-dependent desensitization (also referred to as Ca^{2+}-dependent inactivation) (Yamakura and Shimoji, 1999).

NMDARs are ion-channels highly permeable to Na^{+}, K^{+} and most notably Ca^{2+} (Burnashev et al., 1995b;Schneggenburger, 1996;Garaschuk et al., 1996). In normal extracellular solution with a Ca^{2+} concentration of 1.8mM, fractional Ca^{2+} currents through the NR1/NR2A channels are around 11% (Burnashev et al., 1995a). The asparagine residue at the N site in segment M2 of the NR1 subunit that contributes to voltage-dependent Mg^{2+} block also determines the Ca^{2+} permeability of the NMDA receptor channel (Burnashev et al., 1992); conversely, the NR2 subunit does not contribute to the Ca^{2+} permeability. Interestingly, Ca^{2+} ions not only flux through but also block NMDAR channels when the external Ca^{2+} is increased. Ca^{2+} can markedly reduce single channel conductance of NMDARs (Jahr and Stevens, 1993). This Ca^{2+} block is independent of membrane potential, and mutation of the N site asparagines of

the NR1 or NR2A subunit increases the Ca^{2+} block (Ruppersberg et al., 1993).

Besides the glutamate and glycine binding sites, there are several modulatory sites on NMDARs. NMDARs are blocked by Mg^{2+} in a voltage-dependent manner (Nowak et al., 1984;Jahr and Stevens, 1990). Therefore, activation of NMDARs is also dependent on membrane potential. At resting membrane potentials, NMDAR channels are blocked by physiological concentrations of extracellular Mg^{2+}, but when neurons are depolarized by intense activation of postsynaptic AMPARs, the voltage-dependent block by Mg^{2+} can be relieved. These properties render NMDARs a co-incidence detector for synaptic release of glutamate and depolarization. In addition to Mg^{2+}, several endogenous allosteric modulators of NMDARs have been identified. For example, NMDARs are sensitive to inhibition by proton (H^{+}), and Zn^{2+} can serve as an allosteric modulator of NMDARs as well. Other substances that exert allosteric modulations on NMDARs include polyamines and redox agents.

Although NR1 subunit is indispensable for functional NMDARs (Forrest et al., 1994); (Fukaya et al., 2003), it is the NR2 subunit that confers distinct properties to the receptors (Yamakura and Shimoji, 1999;Cull-Candy et al., 2001). NMDARs containing different NR2 subunit differ in their sensitivity to endogenous and exogenous ligands, permeation and blockage by divalent ions, kinetic properties, and interaction with intracellular proteins (Table 2). For example, the deactivation time course of NR1/NR2A is 6-fold faster than that of NR2B-containing NMDARs. This suggests that NR1/NR2B receptors, once activated, may stay open for a much longer time than those receptors containing NR2A. It is also noteworthy that not many NMDAR antagonists are subunit-specific except highly selective antagonists for

NR2B such as Ifenprodil and Ro 25-6981. To date antagonists with high selectivity for NR2C and NR2D have not been identified. Only recently has a relatively selective antagonist for NR2A, NVP AAM077 (NVP) been developed (Auberson et al., 2002; (Liu et al., 2004a), but its selectivity is still controversial (Berberich et al., 2005; Weitlauf et al, 2005).

Table 2. Differential properties of NMDAR subtypes *

Properties	NMDAR subtypes			
	NR1/NR2A	**NR1/NR2B**	**NR1/NR2C**	**NR1/NR2D**
EC50 of endogenous agonists				
L-Glutamate (μM)	1.7	0.8	0.7	0.4
Glycine (μM)	2.1	0.3	0.2	0.1
Desensitization				
Glycine-independent form	Prominent	Less prominent	Absent	Absent
Ca^{2+}-dependent form	Present	Slight/absent	Slight/absent	Present
Electrophysiological properties				
Single-channel conductance (pS)	40 or 50	40 or 50	22 or 36	16 or 35
Deactivation (τ_w, ms)	50	300	280	1700
Allosteric modulations				
Sensitivity to Mg^{2+} block	High	High	Low	Low
IC50(pH) for proton	7.2	7.3	6.2	7.3
IC50 for Zn^{2+} (μM)	0.005-0.08	0.5-10	14-38	14
Specific antagonists	NVP AAM077 (?)	Ifenprodil Ro 25-6981 CP 101,606	Unidentified	Unidentified

τ_w : Weighted deactivation time constant

***: Adapted from (Cull-Candy et al., 2001;Yamakura and Shimoji, 1999).**

1.6.2. Current understanding of the role of NMDARs in ischemic neuronal injury

It is estimated that the normal extracellular glutamate in the brain is about 1-5 μM (Wahl et al., 1994). Following the onset of stroke, glutamate levels increase dramatically (Nishizawa, 2001). Within 1-2 min after global ischemia, extracellular glutamate begins to increase and it can rise to 16-30 μM by 10-15 min. During focal ischemia, extracellular glutamate also increases quickly, with concentrations varying between 30-50 μM in the ischemic regions (Lipton, 1999a). Results from human stroke patients also support this observation (Castillo et al., 1997). The mechanisms of this glutamate accumulation include enhanced efflux of glutamate and reduced glutamate uptake. The excessive release of glutamate immediately after the onset of ischemia is triggered by activation of voltage-dependent calcium channels and ensuing Ca^{2+} influx (Silver and Erecinska, 1990). With the progress of ischemia, glutamate transporters may operate in reverse mode due to the imbalance of Na^{+} across plasma membranes, which exacerbates the accumulation of extracellular glutamate (Barbour et al., 1988;Taylor et al., 1995); however, it is also demonstrated that glutamate accumulation can be cleared rapidly in global ischemia. In transient focal ischemia, the high glutamate levels do not last for a long time, either. Following reperfusion, glutamate concentrations decline to control (basal) levels quickly, usually less than 1 hour (Nishizawa, 2001;Obrenovitch and Richards, 1995;Davalos et al., 1997) .

The neurotoxic effects of glutamate were first discovered 5 decades ago by Lucas and Newhouse (LUCAS and NEWHOUSE, 1957), and Olney confirmed this glutamate

toxicity (Olney and Sharpe, 1969;Olney, 1969) and coined the term "excitotoxicity". It is now thought that excitotoxicity during stroke is caused by excessive release of glutamate which in turn lead to over-stimulation of glutamate receptors, especially NMDARs. Since the early studies showing that glutamate and NMDARs play a crucial role in hypoxic/ischemic injury *in vitro* and *in vivo* (Choi and Rothman, 1990;Kass and Lipton, 1982;Rothman, 1983;Simon et al., 1984), NMDAR-mediated excitotoxicity has been widely regarded as the major mechanism by which ischemic brain damage occurs.

The signaling pathways linking NMDAR overactivation to neuronal death have caught much attention over the past years. As discussed above, excessive Ca^{2+} enters into neurons through NMDARs following stroke onset, which can activate calmodulin and then nNOS (Dawson and Dawson, 1996). At excitatory synapses of central neurons, NMDARs interact with multiple scaffolding and signaling proteins, such as PSD-95, PSD-93 and SAP102, within the PSD, a microscopic structure associated with the postsynaptic membrane (Sheng and Pak, 2000). For example, the cytoplasmic carboxyl terminals of NR2A and NR2B subunits have been shown to bind to the second PDZ (PSD-95, Dlg and ZO-1 homology) domain of PSD-95 (Kornau et al., 1995). PSD-95 also interacts with nNOS through its second PDZ domain (Brenman et al., 1996;Christopherson et al., 1999); (Komiyama et al., 2002;Kim et al., 1998). Therefore, PSD-95 is thought to play a central role in coupling the over-activation of NMDAR to NO production, and hence neuronal death; however, the exact mechanisms of NMDAR-mediated neuronal death are still not fully understood.

Interestingly, the NR2 subunits of NMDARs may interact differentially with downstream signaling proteins. While calcium-calmodulin kinase II (CaMKII) are associated with both NR2A and NR2B, only its binding to NR2B but not NR2A locks CaMKII in an activated state (Bayer et al., 2001;Strack et al., 2000;Leonard et al., 1999;Gardoni et al., 1998;Strack and Colbran, 1998). NR2B can interact directly with RasGRF1, a Ca^{2+}/calmodulin-dependent Ras-guanine-nucleotide-releasing factor whereas NR2A cannot (Krapivinsky et al., 2003). PSD-95 can bind to both NR2A and NR2B (Niethammer et al., 1996a;Aarts et al., 2002), but it seems that NR2B may preferentially bind to SAP102 (van et al., 2004). These subunit-specific interactions may lead to differential effects following NMDAR activation.

In summary, the role of glutamate and NMDARs in ischemic neuronal death is clear, but further investigations are needed to elucidate the precise mechanisms.

1.7. Current status of NMDAR antagonists in the treatment of stroke

In light of the importance of NMDARs in ischemia-induced neuronal injury, NMDAR antagonists have been considered to be promising neuroprotectants for stroke, and subsequently many drugs have been developed for this purpose.

To date NMDAR antagonists that have been identified can be divided into four types according to their pharmacological properties. The first type is the competitive antagonists; the prototype compounds are 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) but its derivatives d-CPPene and CGS 19755 (Selfotel) and 2-amino-5-phosphonopentanoic acid (AP-5 or APV) and 2-amino-7-phosphonoheptanoic acid (AP-7) are more widely used (Dingledine et al., 1999;Wood

and Hawkinson, 1997). Other competitive NMDA antagonists developed include MDL-100,453, CGP-40116, WAY 126090, N7, NPC17742 and SYM-2351 (De et al., 1999). The most recently developed NR2A specific antagonist NVP AAM077 has also been shown to be a competitive antagonist (Auberson et al., 2002). A subgroup of competitive antagonists target glycine (co-agonist) binding site on NMDARs, including Licostinel (ACEA-1021), Gavestinel (GV-150526), Harkoeride, MDL 29951, ZD 9379, MRZ 2/576 and L689,560 (Jansen and Dannhardt, 2003;Coyle and Tsai, 2004;Coyle et al., 2002).The second type of NMDAR antagonists is the noncompetitive antagonists. Most NR2B-specific NMDAR antagonists are noncompetitive. Ifenprodil and its analogs, including eliprodil and haloperidol, have drawn the most attention (Lynch and Gallagher, 1996;Gallagher et al., 1996). Derivatives of ifenprodil with higher selectivity such as Ro 25-6981, Ro 8-4304 and CP-101,606 have also been developed (Menniti et al., 1997;Fischer et al., 1997b;Kew et al., 1998). Other less commonly known noncompetitive antagonists include ethanol (at intoxicating concentrations) and dynorphin peptides (Dingledine et al., 1999). The uncompetitive antagonists are the third type of NMDAR antagonists. They act only on the activated (open) receptors but not the receptors at rest. The unique property of these antagonists is that they exert a state-dependent and use-dependent block, i.e., they will not reach the binding site unless the ion channel is open and the Mg^{2+} block is removed (Dzubay and Jahr, 1996;Jahr, 1992). For this reason, these drugs are also known as open channel blockers. Once they are bound, these blockers can be trapped by channel closure and recovery from the trapped blocked state is generally slow. Early agents of this type include dizocilpine (MK801), phencyclidine (PCP) and its

derivatives N-ethyl-1-phenylcyclohexamine (PCE) and 1-[1-2(thienyl)cyclohexyl]-piperadine (TCP) (Dzubay and Jahr, 1996;Jahr, 1992;MacDonald et al., 1991;MacDonald et al., 1990). Other drugs that have also been well studied include aptiganel (CNS1102, Cerestat), ketamine, amino-adamantane derivatives, such as memantine and amantadine, and MRZ2/579 (Hewitt, 2000;Sareen, 2002;Liu et al., 2000). The fourth type of NMDAR antagonists contains those blocking NMDARs with unknown mechanisms, such as nitrous oxide and some mGluR agonists (Dingledine et al., 1999).

Since the seminal work by Simon et al. showing that the competitive antagonist APV protect the brain from ischemic damage (Simon et al., 1984), the effects of a variety of NMDAR antagonists on neuronal injury resulting from hypoxia/ischemia have been extensively studied in both the *in vitro* and *in vivo* systems. In cultured neurons, almost all types of NMDAR antagonists tested have shown neuroprotection to some extent. Goldberg et al first showed that APV and several other NMDAR antagonists were potent neuroprotectants against OGD-induced cell death in cortical cultures (Goldberg et al., 1987). The authors also found that uncompetitive antagonists such as MK801 and PCP blocked neuronal injury triggered by NMDA stimulation and OGD challenge (Goldberg et al., 1988). Excitotoxic neuronal death can be ameliorated by NR2B subunit-specific antagonists such as ifenprodil and Ro 25-6981 as well (Fischer et al., 1997a;Graham et al., 1992). Glycine-site antagonists also exert neuroprotection in cultured cortical neurons (Boireau et al., 1996). The neuroprotective actions of NMDAR antagonists have also been determined using organotypic corticostriatal and hippocampal brain slices (Reyes et al., 1998;Calabresi

et al., 2003).

The neuroprotection of NMDAR antagonists observed in the hypoxia models *in vitro* have been confirmed in many animal studies as well. In transient or even permanent focal ischemia model, there is consistent evidence showing reduction in infarct size and improvement in neurological function by all types of antagonists. When administered before or during a temporary ischemia, almost all of the NMDAR antagonists are very effective. Some studies also reported that administration of NMDAR antagonists following focal ischemia can still provide neuroprotection, albeit in a somewhat less dramatic way (Bar-Joseph et al., 1994;Warner et al., 1991). NMDAR antagonists seem to protect the penumbra only in focal ischemia, and the core of the lesion cannot be rescued by NMDAR blockade (Lipton, 1999a), however, these postischemic effects are still controversial (Nellgard and Wieloch, 1992). In global ischemia, the neuroprotective effects of NMDA antagonists are still not convincing. The open channel blocker MK801 and other glycine-site antagonists have been reported to show no effects (Warner et al., 1995;Buchan et al., 1991).

Inspired by the huge success in animal studies, Albers et al. proposed that NMDAR antagonists might be ready for clinical trials (Albers et al., 1989). Over the past years, dozens of NMDAR antagonists have entered acute ischemic stroke clinical trials. This long list includes many types of NMDAR antagonists available (De et al., 1999;Bleich et al., 2003). Selfotel, a representative of competitive NMDAR antagonists, proceeded to phase III stroke trial but was halted because of risk/benefit ratio concern and potential neurotoxic effects accompanied with this drug (Davis et al., 2000;Davis et al., 1997). For similar reasons, the phase III clinical trial for

noncompetitive NR2B-specific antagonist eliprodil was terminated (Lees, 1997). Uncompetitive antagonists were considered to be good candidates for stroke therapy, but a carefully designed clinical trial demonstrated that aptiganel hydrochloride (Ceresat, CNS 1102) was not efficacious or may even be harmful (Lees, 1997;Albers et al., 2001). Glycine-site NMDAR antagonists were shown to have fewer side effects in animal studies, but again, to the disappointment of scientists and clinicians, gavestinel failed in clinical trial for stroke (Lees et al., 2000;Sacco et al., 2001).

In conclusion, not a single NMDAR antagonist has shown positive results in human patients.

1.8. Why NMDAR antagonists failed in stroke clinical trials

As discussed, none of the NMDAR antagonists has been successful in clinical trials involving stroke. Whether it is because of the defects of the antagonists per se, the inappropriate timing of treatment with these antagonists or some other unknown mechanisms remains to be determined (Cheng et al., 2004). Given the clear role of NMDARs in excitotoxicity, the failure of such antagonists has long been perplexing. Ikonomidou and Turski (Ikonomidou and Turski, 2002b) recently argued that, since NMDARs mediate the slow component of synaptic transmission, and synaptic transmission is essential for proper functioning of the brain, NMDAR antagonists actually may hinder normal brain function. Moreover, since glutamate levels in the brain may return to normal rapidly (within 1h after stroke onset, (Nishizawa, 2001;Obrenovitch and Richards, 1995;Davalos et al., 1997)), glutamate-induced excitotoxicity may not contribute to brain injury at all after the acute phase of stroke

(within 6 h after stroke onset); however, in a clinical setting most patients will not be able to receive treatment within 6 h. Together, these may explain why NMDAR antagonists failed in all stroke clinical trials.

In fact, despite the overwhelming reports on the neuroprotective effects of NMDAR antagonists on ischemic brain damage, over the past years some studies did indicate that NMDAR antagonists might be deleterious. It has been shown that blockade of NMDARs with MK801 during normoxia can cause acute neuronal damage (Olney et al., 1989;Allen and Iversen, 1990). More recently, Ikonomidou et al demonstrated that the blockade of NMDARs during early development triggers extensive apoptosis in the brain including cortex, hippocampus and thalamus (Ikonomidou et al., 1999b). Therefore, NMDARs may also play a role in neuronal survival. This is further supported by the evidence that stimulation of NMDARs can lead to upregulation of anti-apoptotic proteins of the Bcl-2 family (Zhu et al., 2005), whereas blockade of NMDARs in the developing brain results in impaired Erk activity, and hence apoptosis (Hansen et al., 2004).

Taken together, there is evidence suggesting the dual role of NMDARs in both neuronal survival and death. The key to answer why NMDAR antagonists are not successful in the treatment of ischemic brain injury may lie in the understanding of the mechanisms by which NMDARs function differentially in determining neuronal fate.

1.9. Rationales, hypotheses and specific aims

While the mechanisms of the paradoxical role of NMDARs in excitotoxicity are still

elusive, Hardingdam et al. found recently that stimulation of NMDARs at synapses activates cell-survival gene BDNF expression and prevents neurons from apoptosis, whereas activation of extrasynaptic NMDARs triggers a pro-death signaling pathway that can be overwhelming (Hardingham et al., 2002). Thus, synaptic and extrasynaptic NMDARs seem to play opposing roles in neuronal fate. Furthermore, these differential effects of NMDARs are developmentally regulated (Hardingham and Bading, 2002). Consequently, the different anatomical locations of NMDARs may account for the paradoxical roles of NMDARs in neuronal fate.

However, most recent evidence has shown that synaptic activity can be excitotoxic (Bellizzi et al., 2005b), which makes the site-specific action of NMDARs somewhat questionable. Furthermore, although it is being challenged (Thomas et al., 2006), mounting evidence has shown that synaptic and extrasynaptic NMDARs have distinct subunit composition (Tovar and Westbrook, 1999). NR2A-containing NMDARs may be predominant at synapses while NR2B-containing NMDARs at extrasynaptic sites. Interestingly, recent studies suggest that the subunit composition of NMDARs may dictate their functions. For example, NR2A- and NR2B-containing NMDAR subtypes may play differential roles in mediating synaptic plasticity (Liu et al., 2004a;Massey et al., 2004b), and different NMDAR subtypes may also be involved in different pathological conditions, including some neurodegenerative diseases (Lynch and Guttmann, 2002;Waxman and Lynch, 2005).

In this study, I hypothesized that NR2A- and NR2B-containing NMDARs may have differential roles in supporting neuronal survival and mediating neuronal death. The specific aims of this thesis project are:

Aim 1: Determine if NR2A- and NR2B-containing NMDARs play differential roles in neuronal apoptosis. I will induce NMDAR-mediated apoptosis in mature cortical neurons, which possess mainly NR2A- and NR2B-containing NMDAR subtypes, by relatively mild stimulation with NMDA, and then take advantage of NR2A- and NR2B-selective antagonists to study the individual role of these NMDAR subtypes. If I find these NMDAR subtypes have differential roles in neuronal apoptosis, I will then

Aim 2: Determine if the subcellular (synaptic versus extrasynaptic) location or the subunit composition of NMDARs causes their functional differentiation. I will first determine the subunit composition of synaptic and extrasynaptic NMDARs by electrophysiological methods. Thereafter, the two NMDAR subtypes at either subcellular location will be isolated pharmacologically and the roles of these NMDAR subtypes in neuronal apoptosis be studied. If it is the subunit composition of NMDARs that determines their role in neuronal apoptosis, I will proceed to

Aim 3: Determine if specifically stimulating the pro-survival NMDAR subtype and/or inhibiting the pro-death subtype reduces neuronal apoptosis following stroke *in vitro*. I will adopt the well-established *in vitro* OGD stroke model to achieve this goal. If the results show that OGD-induced neuronal apoptosis can be attenuated by enhancing the survival-promoting and/or antagonizing the pro-apoptotic NMDAR subtype, an *in vivo* study will follow to

Aim 4: Determine if the treatments conducted *in vitro* (Aim 3) will be effective *in vivo*. A well-characterized focal cerebral ischemia model MCAo will be used, and neurological behavior and cerebral infarction will be compared between the treated

and non-treated groups to assess the efficacy of the treatments.

The ultimate goal of this research project is to cast light on the mechanisms of the NMDAR-dependent ischemic brain damage, hence to develop effective NMDAR-based treatment of ischemic brain injury.

CHAPTER 2: MATERIALS AND METHODS

2.1. Drugs and solutions

All chemicals and drugs used were purchased from Sigma unless specifically indicated. The extracellular solution (ECS) were composed of (in mM): 25 HEPES acid, 140 NaCl, 33 glucose, 5.4 KCl 1.3 $CaCl_2$ and 1 $MgCl_2$ (normal ECS). Mg^{2+}-free ECS did not contain $MgCl_2$. Osmolarity of ECS was adjusted to 320–330 mosM and pH to 7.35. Modified RIPA buffer contained: 150 mM NaCl, 50 mM Tris (pH 7.4), 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate (DOC), 1 mM EDTA and 1 mM Na_3VO_4. Protease inhibitors, including 10 μg/ml each of aprotinin, leupeptin (Peptides International) and 1mM phenylmethylsulfonyl fluoride (PMSF), were added before use. PBS contained (in mM): 138 NaCl, 2.67 KCl, 8.1 Na_2HPO_4, 1.47 KH_2PO_4, 0.9 $CaCl_2$ and 0.5 $MgCl_2$. pH was adjusted to 7.4 with HCl. Mg^{2+} and Ca^{2+} free PBS did not contain $MgCl_2$ or $CaCl_2$ (Invitrogen). The glucose-free bicarbonate-buffered solution for OGD experiment contained (in mM): 121 NaCl, 5 KCl, 1 Na-pyruvate, 1.8 $CaCl_2$, 25 $NaHCO_3$, 0.01 glycine. Solutions used in electrophysiological experiments were detailed below (See Electrophysiology).

2.2. Primary culture of cortical neurons

Dissociated cultures of cortical neurons were prepared from 18-day Sprague-Dawley rat embryos (Mielke and Wang, 2005). The cortices were dissected from the brain and collected in a 35mm dish with Mg^{2+} and Ca^{2+} free PBS. The cortices were then digested with 0.5% trypsin in a 5% CO_2 incubator at 37°C for 10 min. After digestion, the neurons

were further dissociated in Neurobasal medium by trituration with a glass pipette. Neurons were then seeded onto 12-well plates or dishes (35mm or 10cm) at a density of 2.5-3.0 x 10^5/ml and grown in Neurobasal medium containing 2% B-27 supplement and 0.5 mM glutamine (all from Invitrogen) at 37°C in a humidified 5% CO_2 atmosphere. To obtain mixed cortical cultures enriched with neurons, uridine (10 μM) and 5-Fluor-2'-deoxyuridine (10 μM) were added to the culture medium at 3 days in vitro (DIV) and maintained for 48 h to inhibit non-neuronal cell proliferation, and then the cultures were shifted back to the regular culture medium. The medium was changed every 4 days. Mature neurons (11-14 DIV) were used for experiments.

2.3. *In vitro* stimulations

NMDA (50 μM) and glycine (10 μM) were bath applied to neurons for 20 min to facilitate induction of apoptosis. To induce apoptosis by staurosporine (STS), neurons were treated with STS (100 nM) for 1 h by bath application. Specific blockade of synaptic NMDARs was achieved by treatment with MK801 (10 μM) in the presence of bicuculline (50 μM) for 10-15 min, followed by thorough wash with normal ECS to remove any trace of MK801. Wherever NR2A-specific NMDAR antagonist NVP AAM077 (NVP, 0.4 μM; generous gift of Dr. Yves P. Auberson, Novartis Pharma AG, Basel, Switzerland) or NR2B-specific NMDAR antagonist Ro 25-6981 (Ro, 0.5 μM) was involved, the antagonist was pre-incubated with neurons for 10 min and maintained at the same concentration throughout the treatments. *In vitro* stimulations were conducted in Mg^{2+} free ECS. Termination of stimulations was achieved by washing 2x with normal ECS. Neurons were then changed back to normal culturing conditions until further assay.

2.4. Assessment of neuronal apoptosis

To visualize apoptotic neurons, Hoechst-33342 (10 μg/ml) was added to the culture medium 20 h after treatments and incubated at 37°C for 45 min. Images of different treatment groups were taken with a fluorescence microscope (Leica DMIRE2, 40x). To quantify neuronal apoptosis, 20 h following treatments, neurons were lysed, centrifuged (200 x g, 10 min) and then the supernatant (20 μl) was transferred into a strepatavidin precoated microplate. Neuronal apoptosis was determined by measuring intranucleosomal DNA fragmentation using a Cell Death Detection ELISA^{PLUS} kit (Roche Applied Science). Data analysis was carried out according to the manufacturer's instructions for the kit.

2.5. Experimental stroke models

2.5.1. OGD *in vitro*

Cortical cultures were transferred to an anaerobic chamber containing a 5% CO_2, 10% H_2, and 85% N_2 (<0.2% O_2) atmosphere (Goldberg and Choi, 1993), and then washed 3× with the glucose-free bicarbonate-buffed solution (deoxygenated in the anaerobic chamber for 30 min before use) and maintained anoxic for 1 h at 37°C in the incubator inside the anaerobic chamber. OGD was terminated by washing the cultures 2× with ECS, and then the neurons were switched back to the original culturing conditions until assaying for cell death.

2.5.2. MCAo in rats

Transient focal cerebral ischemia was produced by MCAo, as previously described (Liu et al., 2000;Longa et al., 1989). Briefly, male Sprague-Dawley rats (Charles River Laboratories) weighing ~300g were fasted overnight but allowed free access to water. Anesthesia was induced and maintained with 4.0% and 1.5% Isoflurane, respectively. MCAo was achieved by introducing a 3-0 monofilament suture to the MCA via internal carotid artery, as previously described (Longa et al., 1989). Body temperature was controlled at 37 ± 0.5°C, and blood pressures and gases were monitored during the experiment.

To study the effects of pretreatment with drugs, the right femoral vein of the rats was cannulized following anesthetization, and a single bolus of vehicle (saline) or drug was given via intravenous injection (i.v.). 30 min (for NVP and Ro) or 1 hour (for Tat-NR2B-AA and Tat-NR2B9c) following injection, the animals were subject to a 1 hour (for NVP and Ro) or 90 min (for Tat-NR2B-AA and Tat-NR2B9c) MCAo. For post-treatment experiments, animals were first subject to a 90-min cerebral ischemia produced by MCAo. To determine the effects of activation of NR2A-containing NMDARs, the animals were then treated 4.5 hour after onset of MCAo with vehicle (saline) or drug(s) (glycine and/or NVP and Ro) through intraperitoneal injection (i.p.). To examine the effects of NR2B C-tail peptides, vehicle (saline) or peptide (Tat-NR2B-AA or Tat-NR2B9c) was given 1 hour after MCAo onset.

Neurological function testing was performed to grade neurological function on a scale of 0 to 12 (normal = 0; worst = 12) 60 min following MCAo onset and/or 10 min before the animals were sacrificed. Specifically, a battery of two tests that have been used

previously to evaluate various aspects of neurological function (De et al., 1989;Bederson et al., 1986) were carried out: (1) the postural reflex test to examine upper body posture, and (2) the forelimb placing test to examine sensorimotor integration in forelimb placing responses to visual, tactile, and proprioceptive stimuli.

24 hours following MCAo, brains were perfusion-fixed with 4% paraformaldehyde, and brain blocks were embedded in paraffin. Coronal brain sections (5 μm) were cut and stained with hematoxylin and eosin (H & E). 8 coronal levels throughout the brain were selected and images were taken with a microscope (Zeiss, Axiovert 200, 1.5x). Cerebral infarct area (S) of 8 selected sections (between Bregma +3.2mm and -5.8mm) was traced using the ImageJ software, and then total infarct volume (V) was calculated using the following formula:

$$\sum_{i=1}^{n} V_i = (S_{i-1}+S_i)*d_{i-1}/2,$$

where n=9, S_i represents the infarct area of a selected brain section i, Vi denotes the infarct volume between section S_{i-1} and S_i, d is the distance between two adjacent brain sections, $S_0=0$ and $S_9=0$.

2.6. Electrophysiology

2.6.1. Recording of miniature postsynaptic currents (mEPSCs)

Recording of mEPSCs was done on 11-day old cultured cortical neurons. The neurons on coverslips were transferred to a recording chamber that was continuously perfused with ECS. In addition, bicuculline (10 μM) and tetrodotoxin (0.5 μM; Alomone) were added to the ECS to block gama-aminobutyric acid A type ($GABA_A$) receptors and voltage-gated sodium channels, respectively, to isolate action potential-independent

mEPSCs. Patch pipettes were pulled from borosilicate glass capillaries (Sutter Instrument) and filled with an intracellular solution (pH 7.2; 300-310 mOsm) composed of (mM): 140 CsCl gluconate, 0.1 $CaCl_2$, 10 HEPES, 2 $MgCl_2$, 10 BAPTA, and 4 ATP. A MultiClamp 700A amplifier (Axon Instruments) was used for the recording. The series resistance was monitored throughout each recording so that recordings with series resistance varied by more than 10% were rejected. No electronic compensation for series resistance was employed. Whole-cell patch-clamp recordings were performed in voltage-clamp mode while maintaining the membrane potential at -60 mV. Recordings were low-pass filtered at 2 kHz, sampled at 10 kHz, and stored in a PC using Clampex 8.0 (Axon). Synaptic events were analyzed offline using the Mini Analysis Program 6.0 (Synaptosoft).

The removal of extracellular Mg^{2+} eradicates the Mg^{2+}-mediated blockade of NMDARs so that mEPSCs comprising both AMPA and NMDA receptor-mediated components can be measured. Antagonist for NMDARs (Ro or APV (Tocris)) was bath applied for at least 10 minutes to obtain sufficient length of recording for analysis after achieving a stable level of NMDAR blockade. Synaptic events before and after application of NMDAR antagonists were automatically detected from computer stored recordings using the same detection parameters in Mini Analysis Program. Subtraction of averaged traces was done in Excel (Microsoft).

2.6.2. Recording of NMDA induced current mediated by extrasynaptic NMDARs

Extrasynaptic NMDARs were isolated by specifically blocking synaptic NMDARs using open channel blocker MK801 (10 μM, Tocris). To ensure all synaptic NMDARs are activated during the MK801 treatment, cortical neurons were treated with high

concentration of bicuculline (50 μM) to enhance the excitatory synaptic inputs for 15 minutes before and during the MK801 treatment (10 min). After extensive wash by normal ECS to remove any trace of MK801 that is not trapped in opened NMDARs, the coverslip with treated cortical neurons was transferred to a recording chamber for whole-cell patch clamp recording. Extrasynaptic NMDARs in voltage-clamped cortical neurons were activated by NMDA (200 μM) using a fast perfusion system (Warner).

2.6.3. Recording of field EPSCs (fEPSCs) in hippocampal slices

Acute hippocampal slices (400 μm) were prepared from SD rats of 20-36 days old using a vibratome. Hippocampal slices were perfused at room temperature with artificial cerebrospinal fluid (ACSF) containing (in mM) 126 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.2 KH_2PO_4, 26 $NaHCO_3$ and 10 glucose and bubbled with 95% O_2 and 5% CO_2. Field potentials were recorded with glass micropipettes (2-4 MΩ) filled with ACSF placed in the striatum radiatum 60-80 μm from the cell body layer. Synaptic responses were evoked by stimulation (0.05 ms) of the Schaffer collateral-commissural pathway with a bipolar tungsten electrode in the presence of bicuculline methiotide (10 μM).

2.7. Peptide construction and delivery

All the peptides used were rendered cell-permeant by fusing each to the cell-membrane transduction domain of the human immunodeficiency virus-type 1 (HIV-1) Tat protein (YGRKKRRQRRR) (Schwarze et al., 1999). The wild-type NR2B peptide (Tat-NR2B9c) is a fusion protein comprising Tat and the last 9 residues of the NR2B subunit (KLSSIESDV). The control peptides for NR2B9c include: Tat38-48 (comprising HIV-1

Tat residues 38-48 outside the transduction domain), Tat-AA (comprising Tat and two alanine residues), and Tat-NR2B-AA (containing the nine amino acids of the COOH-terminal of NR2B but with a double point mutation in the PSD-95 binding motif, KLSSIEADA). The pTat-PDZ1-2 and pTat-GK fusion proteins were generated by insertion of PSD-95 residues 65-248 encoding the first and second PDZ domains (PDZ1-2), residues 534-724 encoding the guanylate kinase-like domain, respectively, into pTat-HA plasmids. Fusion proteins contained a 6X His-tag, Tat and a hemaglutinin-tag N-terminal to the insert. Plasmids were transformed into BL21 (DE3) LysS bacteria (Invitrogen) and recombinant proteins were isolated under denaturing conditions on a Nickel-His column.

To visualize the delivery of Tat-NR2B9c into the brain, fluorescent Tat-NR2B9c-dansyl and cell-impermeant Tat38-48-dansyl were injected (i.p.) into C57BL/6 mice (25 g) (3 nmol/g). 1 hour later, the animals were perfused with fixative solution (3% paraformaldehyde, 0.25% glutaradehyde, 10% sucrose, 10 U/ml heparin in saline). Brains were removed, frozen in 2-methylbutaneat (-42 °C) and 40 μM sections were cut with a cryostat. Coronal sections were examined for dansyl fluorescence by UV-laser confocal microscopy.

2.8. Construction of cDNAs of NR2A and NR2B C-terminals

Two pairs of primers, (5')CGCGGATCCGAGCACCTCTTCTACTGGAAG(3'), (5') GGCGGATCCTTAAACATCAGATTCGATAC(3') and (5')CGCGGATCCGAG-CATCTGTTCTATTGGCAG(3'), (5')CGCGGATCCTCAGACATCAGACTCAATAC (3') were used to amplify the C-terminals of NR2A (residues of 837 to 1465) and NR2B

(residues 838 to 1482), respectively. The PCR amplified C-terminal of NR2A or NR2B was then inserted into the BamHI sites of mRFP-C1 expression vector. Sequencing was performed to confirm the constructs.

2.9. RNAi (RNA interference)

The siRNAs for RNAi comprised 19 nucleotides with a 2-nucleotide 3' overhang. The duplex for silencing NR2A mRNA (nucleotides 3182-3200) was: sense: (5') CUCUCAAUGAGUCCAACCCAA (3'), with a phosphate group at the 5' end; anti-sense: (5') GGGUUGGACUCAUUGAGAGUG (3'). The duplex designed to target NR2B mRNA (nucleotides 2983-3001) was: sense: (5') AGGAGCGCCAAUCCGUGAUCU (3'), with a phosphate group at the 5' end; anti-sense: (5') AUCACGGAUUGGCGCUCCUCU (3'). siRNAs were synthesized by Qiagen (Ontario, Canada). Neurons (DIV 9) cultured on 35mm dishes were transfected with either NR2A or NR2B siRNAs using the Transmessenger Transfection kit (Qiagen) and following the manufacturer's suggestions. Briefly, diluted Enhancer R in the appropriate volume of Buffer EC-R and then added siRNA (the ratio of nucleic acids to Enhancer R was 1:8). The siRNA-Enhancer R mixture was incubated briefly (2-5min) at room temperature. TransMessenger Transfection Reagent was added to the mixture and incubated for another 10 min at room temperature to allow formation of the nucleic acid-TransMessenger Reagent complexes. Diluted the complexes with equal volume of culture medium and added drop-wise to the neurons. After incubation for 3 hours under normal growth conditions, neurons were washed 1x with PBS, and maintained under original culture conditions for 48 h before further analysis.

2.10. Transfection

cDNAs of NR2A or NR2B carboxyl tail were transfected into neurons (DIV 9) cultured on 1.8 cm coverslips using the CalPhos mammalian transfection kit (Clontech). Briefly, for each coverslip, 2 μg of cDNA was first mixed with $CaCl_2$ solution, and then added to HEPES-buffered saline. This transfection solution was added drop-wise to the neurons. After incubation for 1-3 hours in a CO_2 incubator at 37 °C, the transfection was terminated by further incubation with culture medium (pre-equilibrated in a 10% CO_2 incubator) for 20 min. The coverslips were then transferred back to the original culture medium and maintained under normal growth conditions. 48 h later, the transfection efficiency for neurons were examined.

2.11. Western blot

For detection of Akt phosphorylation and caspase 3 activation, neurons were lysed using modified RIPA buffer in the presence of protease inhibitors 12 hours after treatments, and proteins were extracted. 40 μg of total proteins were subjected to 10% and 15% SDS–PAGE for probing of phospho-Akt and cleaved caspase 3, respectively, and then immunoblotted with respective antibodies according to the manufacturer's instruction. Except for the β-tubulin antibody (Sigma), antibodies for phospho-Akt (Ser473), Akt and cleaved caspase 3 (Asp175) were purchased from Cell Signaling. For sequential re-probing of the same blots, the membranes were stripped of the initial primary and secondary antibodies and subjected to immunoblotting with another antibody. To detect NR2A and NR2B expressions after RNAi, proteins were extracted for western blotting 48 hours after siRNA transfection, and 20 μg of total protein were separated on 8% SDS–

PAGE gels and probed with anti-NR2A or -2B antibody (US Biological). Detection of proteins was achieved using horseradish peroxide (HRP)-conjugated secondary antibodies and enhanced chemiluminescence (Amersham).

2.12. Co-immunoprecipitation (Co-IP)

Rat brain proteins were prepared under weakly denaturing conditions known to permit NMDAR/PSD-95 interaction. Adult Wistar rat forebrains were removed and homogenized in ice-cold buffer (0.32 M sucrose, 0.1mM Na_3VO_4, 0.02 M p-nitrophenyl phosphate, 0.02 M glycerol phosphate, 0.1 mM PMSF, and 5 μg/ml each of antipain, aprotinin, and leupeptin). Homogenates were centrifuged at 800 x g for 10 min at 4 °C. The supernatants were centrifuged at 11,000 x g at 4 °C for 20 min and the pellets (P2) were resuspended in homogenization buffer. P2 membranes, a fraction enriched with synaptic structures, were adjusted to 200 μg protein/90 μl with homogenization buffer, and DOC and Triton X-100 were added to final concentrations of 1% and 0.1% respectively. After 30 min at 37 °C, suspensions were centrifuged at 100,000 x g_{av} for 10 min and the supernatants were used for co-IP. Tat peptides (100 μM) were incubated with 200 μg of rat forebrain lysate for 1 hour at 37 °C. Following the protocol described above, PSD-95 was precipitated from rat forebrain extracts using anti-NR2A and NR2B antibodies, which were generated against the amino acid residues 934-1203 of the NR2A protein and residues 935-1455 of the NR2B protein, respectively. Proteins were then separated on 8% SDS-PAGE gels and probed with the appropriate antibodies (See Western blot).

2.13. Calcium imaging

Ratiometric measurements of free intracellular calcium concentration $[Ca^{2+}]i$ were performed in primary cultured neurons loaded with the fluorescent Ca^{2+} indicator fura-2/AM. NMDA (250 μM dissolved in Mg^{2+}-free solution) was applied by pressure injection (1 s, every 90 s) from a micropipette placed near the cell soma. Evoked calcium responses were recorded before and after bath application of Tat-NR2B9c (50 nM, 20 min). Rate of $[Ca^{2+}]i$ increase was calculated from the time taken to rise from 5% to 95% of peak values. For each cell, peak ratio and rate of $[Ca^{2+}]i$ was averaged from 4 responses obtained before and 4 after Tat-NR2B9c application. Data were expressed as before:after ratios of responses obtained before and after peptide application.

2.14. Data Analysis

Data were expressed as Mean ± SEM where appropriate. Relative difference in apoptosis was calculated by normalizing the acquired absorbance readings to control (set as 100%) and then subtracting control from all the normalized values. Analysis of Variance (ANOVA) was used for comparison among multiple groups, followed by Holm-Sidak test for comparison between two groups. Statistical significance was defined as $p<0.05$.

CHAPTER 3: RESULTS

Part I: NR2A- and NR2B-containing NMDAR subtypes have opposing roles in excitotoxicity

3.1.1. Summary

NR2A- and NR2B-containing NMDAR subtypes may dictate the polarity of synaptic plasticity (Liu et al., 2004a;Massey et al., 2004b); however, whether they play differential roles in excitotoxicity is unknown. In this study, I investigated the roles of these two major NMDAR subtypes in mature cortical neurons in neuronal apoptosis. I found that, while NR2B-containing NMDAR subtype mediates neuronal apoptosis, NMDARs containing NR2A play an opposing role, i.e. activation of NR2A is anti-apoptotic. The distinct roles of these two NMDAR subtypes are not affected by their subcellular (synaptic versus extrasynaptic) location. Taken together, NR2A- and NR2B-containing NMDAR subtypes may play opposing roles in excitotoxicity. These discoveries suggest that general NMDAR antagonism may hinder the pro-survival action of NR2A-containing NMDARs and cause neuronal death, and hence may explain, at least partially, the failure of NMDAR antagonists in the treatment of stroke-related brain injury in clinical studies and lay the foundation for developing novel NMDAR-based therapies for stroke.

3.1.2. NR2A- and NR2B-containing NMDARs play differential roles in neuronal apoptosis

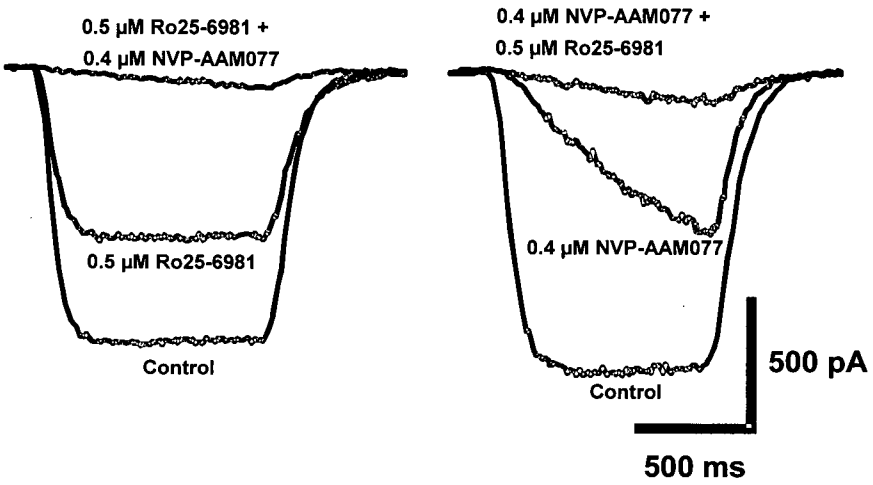
3.1.2.1. NVP AAM077 shows a relatively high selectivity for NR2A-containing NMDARs in the present study

In the adult forebrain, the two major NMDAR subtypes are NR2A- and NR2B-containing NMDARs. To distinguish the roles of these NMDAR subpopulations in neuronal apoptosis in mature cortical neurons, we took advantage of two subunit-specific NMDAR antagonists, NVP AAM077 (NVP) and Ro 25-6981 (Ro) for NR2A- and NR2B-containing NMDARs, respectively. Ro is highly selective for NR2B-containing NMDARs and has been widely used in many studies to identify the unique properties of NR2B-containing NMDARs. The specificity of the newly developed NR2A antagonist NVP, however, has been under debate since its discovery. On one hand, Auberson and colleagues (Auberson et al., 2002) showed that NVP has a 130-fold preference for NR2A relative to NR2B. This relatively high selectivity was also observed in other studies (Liu et al., 2004a;Massey et al., 2004b). On the other hand, Feng et al. found that NVP not only has a poor selectivity for NR2A (only 13-fold over NR2B), but also acts as a potent antagonist for NR2C- and NR2D-containing receptors (Feng et al., 2004). More recent studies also suggested that NVP might also inhibit NR2B-containing NMDARs substantially, especially when applied before agonists (Berberich et al., 2005a;Weitlauf et al., 2005b). Due to the questionable selectivity of NVP and the developmental change in NR2A and NR2B expression, we first determined if both subtypes of NMDARs exist in the neurons used in the present study (cortical cultures of 11-14 days *in vitro* (DIV)) and if NVP functions as a NR2A specific antagonist.

To do this, we examined the ability of these antagonists to inhibit whole-cell currents evoked with a rapid and brief application of NMDA (50 μM NMDA, 10 μM glycine, 5 μM strychnine). As shown in Fig. 1a, bath application of either NVP (0.4 μM) or Ro (0.5 μM) alone produced a partial, but significant, blockade of the NMDA-induced currents. To determine the potentially non-selective, overlapping blockade of these antagonists, we applied the two antagonists sequentially and compared the degree of blockade produced by each antagonist when it was applied alone and following the blockade by the other antagonist. As shown in Fig. 1a and b, NVP produced similar blockage when applied either alone (42.9% ± 5.9%) or after blockade with Ro (43.4% ± 12.4%), confirming that at the respective concentrations used here, Ro is a very specific antagonist to NR2B subunit with little effect on NR2A-containing receptors (Mutel et al., 1998;Fischer et al., 1997a) and NVP can effectively block NR2A-containing receptor-mediated current (Liu et al., 2004a;Massey et al., 2004b;Tigaret et al., 2006a). However, following NVP blockade, the percentage of NMDA current inhibition by Ro was reduced proximately by 5.6% (34.6% ± 1.8% when applied alone vs 29.0% ± 3.3% after NVP, $P>0.05$). Although the reduction is not significant, it may reflect a small degree of cross-inhibition of NR2B receptors by NVP in these neurons under our experimental conditions, as suggested by several recent studies (Weitlauf et al., 2005a;Berberich et al., 2005b;Tigaret et al., 2006b). However, several recent studies have demonstrated that such a small percentage of contaminant NR2B inhibition does not significantly affect the utility of NVP as an NR2A-subunit preferential antagonist under experimental conditions such as in the present study (Liu et al., 2004b;Massey et al., 2004a;Tigaret et al., 2006c). Together, our results indicate that both NR2A- and NR2B-containing receptor subtypes

are expressed in these neurons and under our experimental conditions, the two antagonists selectively block the expected receptor subtypes with little cross-receptor subtype antagonism.

1a


0.5 µM Ro25-6981 +
0.4 µM NVP-AAM077
0.5 µM Ro25-6981
Control
0.4 µM NVP-AAM077 +
0.5 µM Ro25-6981
0.4 µM NVP-AAM077
Control
500 pA
500 ms

1b

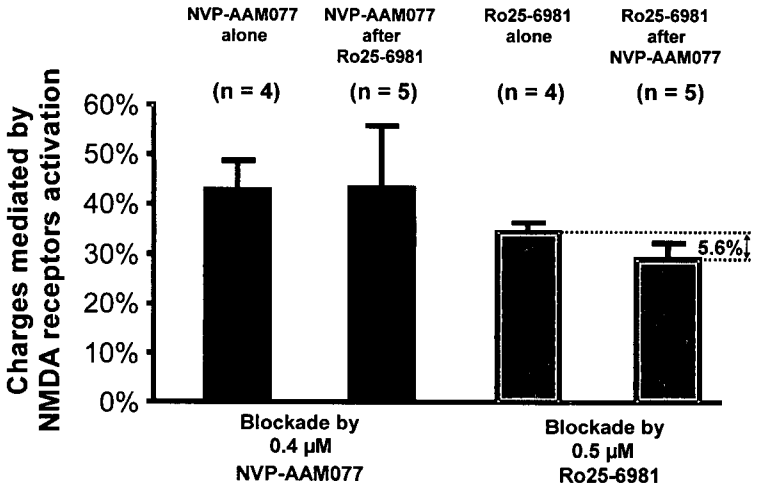

NVP-AAM077 alone
NVP-AAM077 after Ro25-6981
Ro25-6981 alone
Ro25-6981 after NVP-AAM077
(n = 4)
(n = 5)
(n = 4)
(n = 5)
Charges mediated by NMDA receptors activation
60%
50%
40%
30%
20%
10%
0%
5.6%
Blockade by 0.4 µM NVP-AAM077
Blockade by 0.5 µM Ro25-6981

Fig. 1. **Functional NR2A and NR2B-containing NMDA receptors are present in cultured neurons and are preferentially blocked by their respective antagonists. Whole cell recording was performed at a holding membrane potential of -60 mV in an extracellular solution supplemented with 10μM CNQX, 0.5μM TTX, 10μM bicuculline. a. example traces of whole-cell currents evoked by a brief perfusion of 50μM NMDA (plus 10μM glycine and 5μM strychnine) from a multi-barrel fast perfusion system in the absence or presence of specific NR2A- (NVP, 0.4 μM), or NR2B-antagonists (Ro, 0.5 μM) or both. The percentage blockade of the NMDA-induced currents by sequential application of these two antagonists is summarized in histogram in b. Pre-application of Ro did not alter the percentage blockade produced by NVP (p = 0.97; in the absence vs in the presence of Ro), whereas pre-treatment of NVP produced a small, albeit statistically non-significant, reduction in the percentage blockade of the currents produced by Ro (5.6%; p=0.19).**

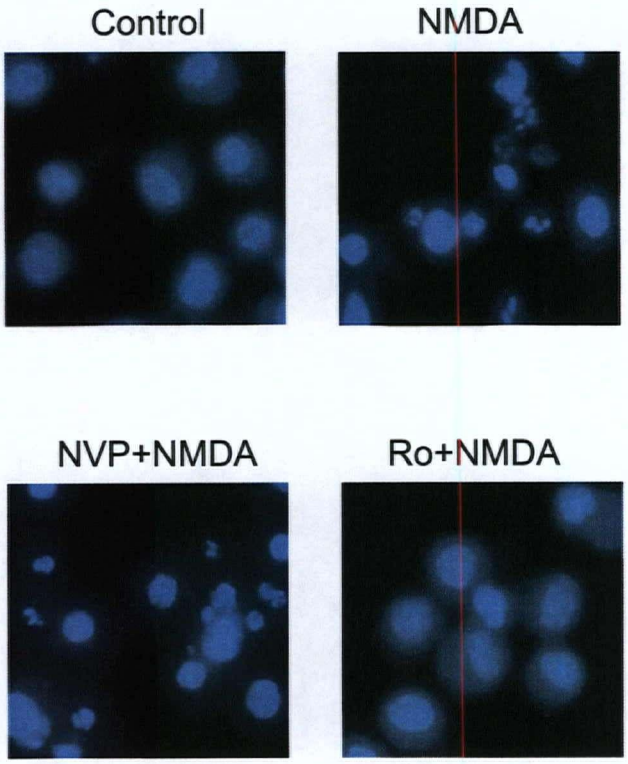
3.1.2.1. Determination of the roles of NR2A- and NR2B-containing NMDARs in neuronal apoptosis using subunit-specific antagonists

Different NR2 subunits confer distinct electrophysiological and pharmacological properties on NMDARs and couple them with different intracellular signaling pathways. Previous studies indicate that NR2A- and NR2B-containing NMDAR subtypes may have opposing roles in dictating the direction of synaptic plasticity (Liu et al., 2004a); (Massey et al., 2004b). To test the hypothesis that NR2A- and NR2B-containing NMDARs have differential roles in neuronal death, I first examined their roles in NMDA-induced neuronal apoptosis using mature rat cortical cultures of 11-14 days *in vitro* (DIV) in which NR2A- and NR2B-containing receptors are the two major NMDAR subtypes.

NMDAR-mediated neuronal apoptosis was induced by briefly incubating neuronal cultures with 50 μM NMDA plus glycine 10 μM for 20 min (NMDA-mediated excitotoxicity). Neuronal apoptosis was determined 20 h after NMDA treatment by nucleus staining with Hoechst 33342, and the degree of neuronal apoptosis was quantified by measurement of intranucleosomal fragmentation. To dissect the roles of NR2A- and NR2B-containing NMDARs, either NVP (0.4 μM) or Ro (0.5 μM) was co-applied with NMDA, which resulted in selective activation of NR2B- or NR2A-containing NMDARs, respectively. Consistent with previous reports (Budd and Lipton, 1999;Hardingham et al., 2002;Liu et al., 2004a;Wang et al., 2004), NMDA stimulation produced neuronal apoptosis as indicated by the increase in the proportion of neurons displayed the characteristic morphological changes of apoptosis (chromatin condensation and/or fragmentation, Fig. 2a). The NMDA-induced apoptosis is a result of specific activation of NMDARs, as it is fully blocked by the NMDAR antagonist APV (Wang et

al., 2004). NMDA-induced neuronal apoptosis was abolished when Ro was co-applied with NMDA (Ro+NMDA) but significantly enhanced when NVP was present during NMDA treatment (NVP+NMDA; Fig. 2a and b, $p<0.05$, compared with NMDA alone), suggesting that selective activation of NR2A-containing NMDARs exerts a cell survival effect while NR2B-containing NMDARs are critically involved in mediating neuronal death. The aggravation of NMDA-induced apoptosis in the presence of NVP was not due to a toxic or other non-specific effect of NVP because, as shown later, incubation of NVP alone with cortical cultures for as long as 4 h does not result in neuronal death (Fig. 3c) and furthermore, NVP-induced cell death can be prevented by simultaneous application of Ro (Fig. 3b).

2a



2b

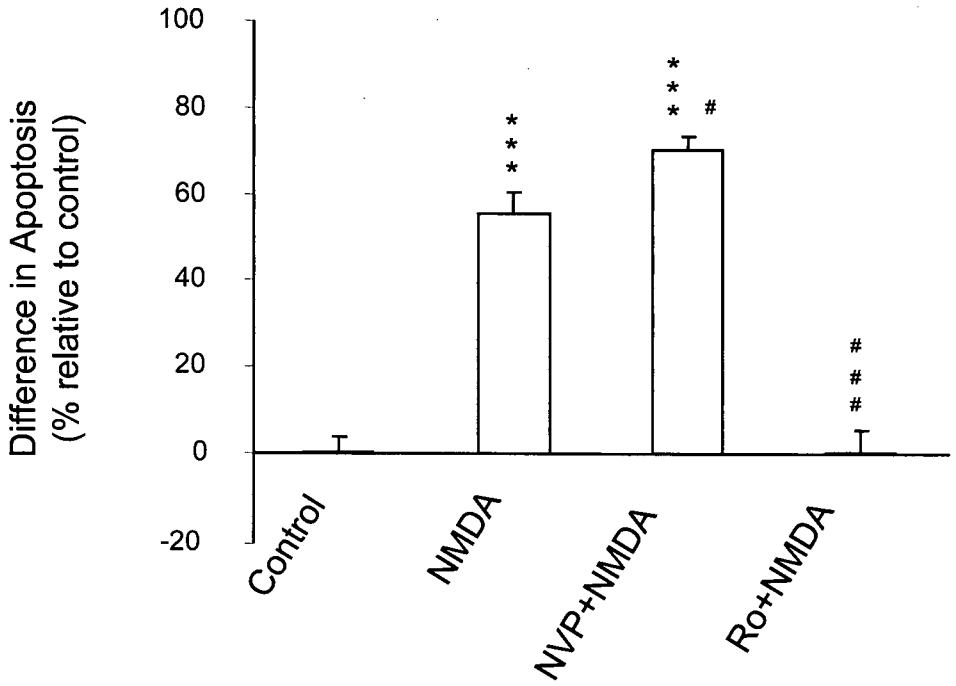

Difference in Apoptosis
(% relative to control)
100
80
60
40
20
0
-20
***
***
#
###
Control
NMDA
NVP+NMDA
Ro+NMDA

Fig. 2a-b. **Activation of NR2A- or NR2B-containing NMDARs exerts differential effects on neuronal apoptosis. a) Representative images illustrate the differential roles of NR2A- and NR2B-containing NMDARs in NMDA-induced neuronal apoptosis. Mature neurons (DIV 11-14) were treated with 50 μM NMDA plus 10 μM glycine for 20 min in the presence of NR2A-specific antagonist NVP-AAM077 (NVP; 0.4 μM) or NR2B-specific antagonist Ro 25-6981 (Ro; 0.5 μM), and stained with Hoechst-33342 20 h following indicated treatments. NMDA stimulation-produced neuronal apoptosis characterized by chromatin condensation and/or fragmentation was aggravated in the presence of NVP (NVP+NMDA), but eliminated in the presence of Ro (Ro+NMDA). b). Quantitative measurement confirmed the differential effects shown in a). Cell death ELISA assay for apoptosis was performed 20 h after the indicated treatments. Data are presented as the difference in apoptosis levels as a percentage of control. *** denotes $p < 0.001$ compared with non-treated control; # and ### denote $p < 0.05$ and $p < 0.001$, respectively, compared with NMDA treatment alone; n = 18 tissue culture wells from three separate experiments for each group.**

3.1.2.2. Examination of the roles of NR2A- and NR2B-containing NMDARs in neuronal apoptosis by RNAi and over-expression of NR2 subunit C-terminal

Pharmacological tools may have limitations when used alone to characterize the function of NMDARs (Neyton and Paoletti, 2006). To confirm that results obtained by using subunit-specific NMDAR antagonists, I attempted to use other methods such as RNA interference (RNAi) and over-expression of the NR2A or NR2B carboxyl tail in neurons. RNAi can silence proteins without knocking out the genes that encode such proteins. Thus, I tried to acutely knock down NR2A or NR2B in cortical cultures using small interfering RNAs (siRNAs) derived from the sequence of NR2A or NR2B mRNA. Unfortunately, in my hands this strategy was not able to specically and efficiently knock down the expression of NR2A or NR2B (data not shown).

The C-terminal of NR2 subunits is very important for the function of NMDARs (Sprengel et al., 1998) since it contains the domains that allow interactions with other intracellular proteins critical for NMDAR signaling (Sheng and Pak, 2000;Kohr et al., 2003;Niethammer et al., 1996b). Therefore, disrupting these receptors from their downstream signaling pathway may be an alternative to pharmacological approaches to examine the functions of NR2A- and NR2B-containing receptors. To do this, I attempted to over-express the C-terminal of NR2A or NR2B in the cortical cultures. The cDNA encoding the full length of NR2A or NR2B C-tail was inserted into the mRFP-C1 expression vector and transfected into neurons (DIV 9-11) using calcium phosphate method (Jiang et al., 2004). Unfortunately, the transfection efficiency of the cDNA of NR2A or NR2B C-tail was near zero (data not shown), rendering it impossible to carry out further experiments.

Because of time limitation, I was not able to optimize the experimental conditions. Nonetheless, both RNAi and over-expression of NR2 subunit C-tail remain potentially good methods to address the issues about the role of NMDAR subtypes in excitotoxicity.

3.1.3. NR2A- and NR2B-containing NMDARs are coupled to distinct signaling pathway

As discussed, cell survival and death are mediated by distinct mechanisms. Thus, we next examined the mechanisms underlying the opposite effects of NR2A- and NR2B-containing NMDARs on neuronal fate. Since phosphatidylinositol 3-kinase (PI3K)-Akt pathway has been shown to promote survival in manifold types of cells including neurons (Brunet et al., 2001;Sutton and Chandler, 2002) whereas caspase 3-dependent apoptotic pathway is activated in NMDAR-mediated neuronal apoptosis (Allen et al., 1999;Tenneti and Lipton, 2000), I probed the levels of phosphorylated Akt and cleaved (activated) caspase-3 12 h after the same treatments as in Fig. 2a and b by Western blotting. Similar to previous reports (Allen et al., 1999;Tenneti and Lipton, 2000;Puka-Sundvall et al., 2000;Sutton and Chandler, 2002;Zhu et al., 2002;Gines et al., 2003), we found that NMDA treatment (50 μM, 20 min) decreased Akt phosphorylation (Ser473) but increased caspase 3 (Asp175) cleavage. When NR2B-containing NMDARs were selectively blocked by Ro (0.5 μM), however, NMDA treatment enhanced Akt and lessened caspase 3 activation, confirming that stimulation of NR2A-containing NMDARs facilitates neuronal survival. Consistent with the effects on neuronal apoptosis, specific activation of NMDARs containing NR2B by blocking NR2A with NVP (0.4 μM) during NMDA treatment further diminished NMDA-induced reduction in Akt phosphorylation while elevated the level of cleaved caspase 3 (Fig. 2c). These findings suggest that the distinct roles of NR2A- and NR2B-containing NMDAR subtypes are probably due to differential downstream signaling. The exact intermediate steps of these signal transduction pathways are yet to be studied.

2c

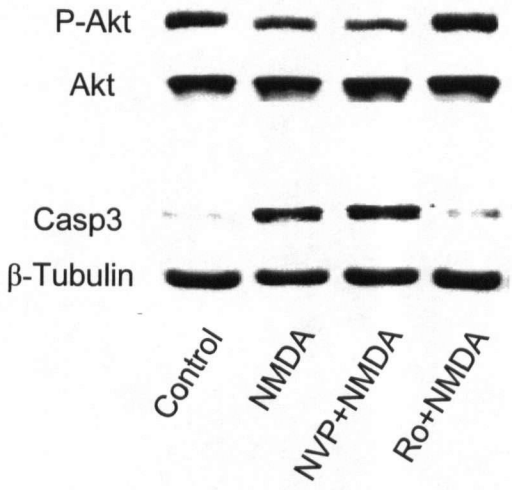


Fig. 2c. **Activation of NR2A- and NR2B-containing NMDARs triggers neuronal survival (Akt) and apoptotic (caspase-3) pathways, respectively. Analysis of phosphorylated Akt (P-Akt; Ser473) and cleaved caspase 3 (Casp 3; Asp175) levels by Western blotting indicates that Akt phosphorylation was increased following stimulation of NR2A-containing NMDARs (Ro+NMDA) but decreased after stimulation of NMDARs containing NR2B (NVP+NMDA). In contrast, caspase-3 cleavage shows a changing pattern opposite to that of Akt phosphorylation.**

3.1.4. The subunit composition rather than the subcellular location determines the role of NMDARs in excitotoxicity

In a recent study, Hardingham et al. reported that selective activation of synaptic and extrasynaptic NMDARs mediates neuronal survival and death, respectively (Hardingham et al., 2002). However, most recent evidence indicated that activation of synaptic NMDARs can lead to neuronal death (Bellizzi et al., 2005a). Furthermore, available immunochemical and electrophysiological evidence suggests that at least in the mature neurons of rat hippocampus and cortex, NR2A- and NR2B-containing receptors are differentially expressed at synaptic and extrasynaptic sites (Tovar and Westbrook, 1999;Stocca and Vicini, 1998). These data raise the possibility that subunit composition, rather than the subcellular (synaptic versus extrasynaptic) location, of NMDARs mediates the opposing actions observed in the present study. To verify such a possibility, we functionally mapped the expressions of NR2A and NR2B-containing NMDARs at synaptic and extrasynaptic sites and investigated their roles in promoting cell survival or death in cultured cortical neurons.

3.1.4.1. Synatpic NR2A- and NR2B-containing NMDARs mediate opposing effects on neuronal fate

3.1.4.1.1. A significant number of NR2B-containing NMDARs exist at excitotary glutamatergic synapses

Although the vast majority of synaptic NMDARs are NR2A-containing, in CA1 neurons in hippocampal slices prepared from rats aged from 3 to 4 weeks, electrophysiological evidence clearly indicates that small proportion of functional NR2B-containing receptors

are expressed at synaptic sites (Liu et al., 2004a). Therefore, we first examined if functional NR2B-containing receptors are also expressed at the synaptic sites of the cultured cortical neurons used in the present study using whole-cell recording of spontaneous mEPSCs. As shown in Fig. 3a, under this recording condition, mEPSCs comprise both fast, AMPAR-mediated component which was completely blocked by non-NMDAR antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) (data not shown), and the slow, NMDAR-mediated component which was fully blocked by NMDAR antagonist APV. Consistent with the presence of certain proportion of functional synaptic NR2B-containing receptors, the NMDA component was significantly reduced by bath application of the specific NR2B-containing NMDAR antagonist Ro (0.5 μM; Fig. 3a-3 and 4). As mEPSCs are primarily mediated by synaptically localized receptors that were activated by glutamate spontaneously released from presynaptic terminals, the sensitivity to NR2B antagonist demonstrates that functional NR2B-containing NMDARs are present within the glutamatergic synapses of the neurons under study. On average, the NR2B-containing receptor-mediated component accounts for 32.4 ± 3.6% of the synaptic NMDA currents (Fig. 3a-3 and 4) and the rest was primarily mediated by NR2A-containing receptors as it was largely eliminated in the presence of NR2A specific antagonist NVP (0.4 μM). Thus, similar to hippocampal CA1 neurons prepared from brain slices (Liu et al., 2004a), both functional NR2A- and NR2B-containing subpopulations of NMDARs, albeit the former being predominant, are expressed at the synapses of the cultured neurons used herein.

3a

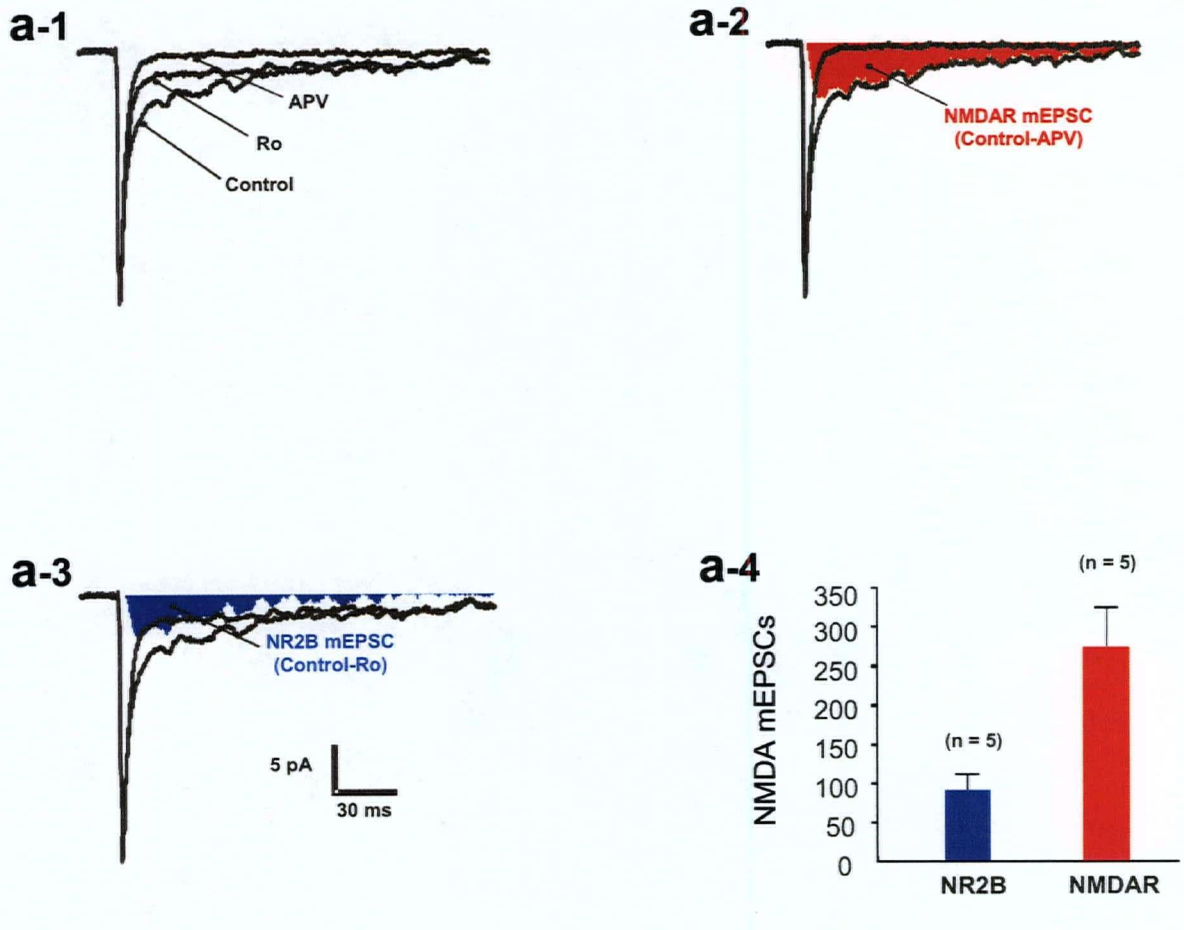

a-1
APV
Ro
Control
a-2
NMDAR mEPSC
(Control-APV)
a-3
NR2B mEPSC
(Control-Ro)
5 pA
30 ms
a-4
(n = 5)
NMDA mEPSCs
350
300
250
200
150
100
50
0
(n = 5)
NR2B
NMDAR

Fig. 3a. **Functional synaptic NR2B-containing NMDARs are present in cultured cortical neurons. Spontaneous mEPSCs were recorded in whole-cell voltage-clamp mode at a holding membrane potential of -60 mV in the presence of tetrodotoxin (0.5 μM) and bicuculline (10 μM) with zero added Mg^{2+}. a-1. Examples of mEPSC traces (averaged from 100 individual events) obtained in the absence (Control) and presence of Ro (0.5 μM) or the broad spectrum NMDAR antagonist APV (APV; 50 μM). a-2. Total NMDAR-mediated component of mEPSCs was obtained by subtracting the averaged mEPSC recorded in the presence of APV from the averaged control mEPSC (Control-APV; shaded region). a-3. The NR2B-containing receptor component was obtained by subtracting the averaged mEPSC recorded in the presence of Ro from the averaged control mEPSC (Control-Ro; shaded region). a-4. Bar graph summarizes data obtained from five individual neurons (n=5). Charge transfer is equivalent to the area of the shaded regions.**

3.1.4.1.2. The pro-apoptotic effect of synaptic NR2B-containing NMDARs is unmasked by blockade of NR2A-containing counterparts

After establishing the presence of both NR2A- and NR2B-containing receptors at the synaptic sites, I examined the function of the two subpopulations of synaptic NMDARs in mediating neuronal survival or death. I reasoned that if the location of the receptors is critical, activation of either receptor population should produce effects of promoting neuronal survival. But, if the subunit composition is the determinant, one would expect that the two populations, although both being synaptically localized, should have opposing actions. To increase activation of synaptic NMDARs by synaptically released glutamate, neurons were incubated with $GABA_A$ receptor antagonist bicuculline (50μM) for 4 hours. Bicuculline increases neuronal excitation by blocking $GABA_A$ receptor-mediated synaptic inhibition and thereby enhances action potential-dependent synchronized release of glutamate from presynaptic terminals (Hardingham et al., 2002). Neuronal apoptosis was quantified 20 h following the treatments. I found that stimulation of synaptic NMDARs by application of bicuculline alone or in the presence of NR2B antagonist Ro (0.5 μM) did not cause apoptotic cell death (Fig. 3b). In contrast, blocking synaptic NR2A-containing receptors by co-application of NVP (0.4 μM) with bicuculline significantly increased neuronal apoptosis ($p<0.001$, Fig. 3b). The NR2A blockade-induced neuronal apoptosis was mediated by synaptic NR2B-containing receptors as it was prevented in the presence of Ro (0.5 μM; Fig. 3b).

3b

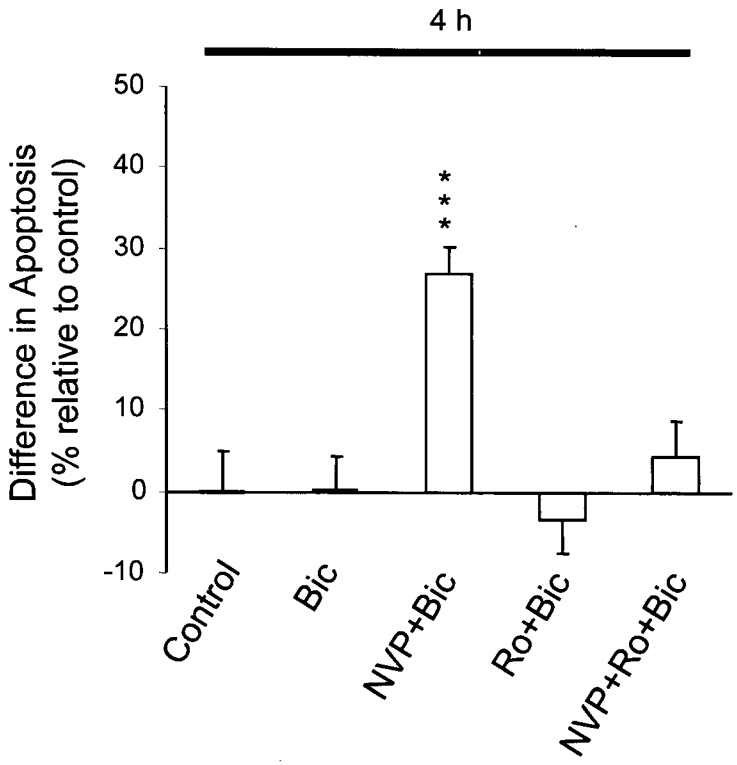
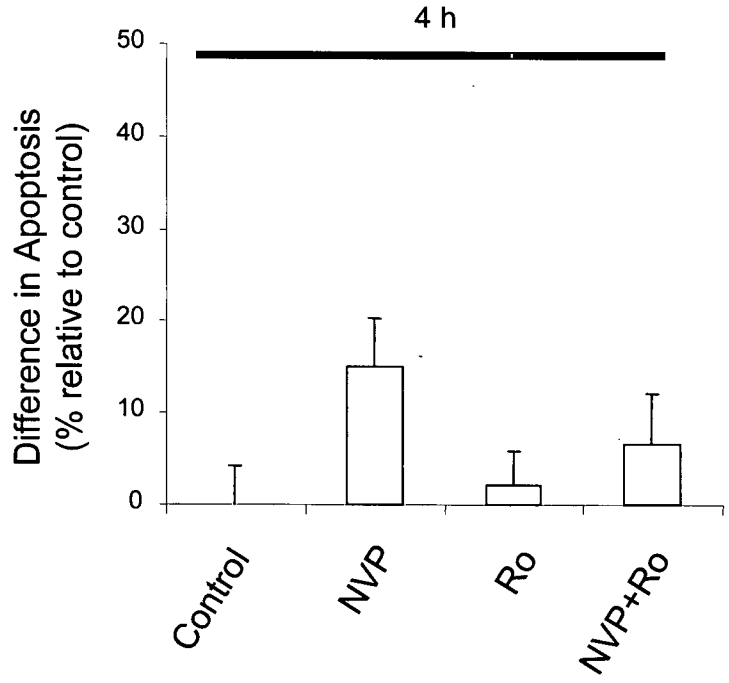

4 h
Difference in Apoptosis
(% relative to control)
50
40
30
20
10
0
-10
***
Control
Bic
NVP+Bic
Ro+Bic
NVP+Ro+Bic

Fig. 3b. **Enhanced activation of synaptic NR2A- and NR2B-containing NMDARs exerts opposing actions on neuronal fate. Potentiation of synaptic NMDAR activation was achieved by increasing the presynaptic release of glutamate through incubation of cultured neurons with bicuculline (Bic; 50 μM) for 4 h in the absence or presence of NR2-specific antagonists. Blockade of NR2A- (Bic+NVP), but not NR2B- (Bic+Ro) containing NMDARs increased neuronal apoptosis. The NR2A blockade-induced apoptosis was prevented by a further blockade of NR2B-containing receptors (Bic+NVP+Ro). *** $p < 0.001$ compared with control; n = 14-16 for each group from three separate experiments.**

Since the increased action potential-dependent synaptic release of glutamate during bicuculline incubation may also activate extrasynaptic NMDARs through glutamate spillover, I next examined the effects of blocking synaptic NMDAR activation by glutamate spontaneously released from terminal under basal, non-stimulated conditions. As shown in Fig. 3c, incubating neurons with NVP (0.4 μM) for 4 h did not cause significant neuronal apoptosis when compared with control cells. These results suggested that NVP has little toxic effect on the cortical cultures and as well confirmed that cell death induced by bath application of NR2A antagonist NVP for 20 min (Fig. 2a and b) is not due to the toxicity of NVP. While a short-term incubation of NVP had little effect on neuronal death, an extended incubation period of 48 h did produce a remarkable increase in neuronal apoptosis (Fig. 3d, $p<0.01$), which was comparable with the level of apoptosis following NVP treatment under bicuculline-stimulated conditions shown in Fig. 3b. In contrast, blockade of synaptic NR2B alone up-to 48 h did not induce any neuronal apoptosis (Fig. 3d). Similarly, the synaptic NR2A antagonist-induced apoptosis was also prevented by the blockade of synaptic NR2B receptors with Ro (0.5 μM). Together, the data strongly suggest that synaptic NR2B-containing NMDARs may mediate neuronal apoptosis, but under physiological conditions, this effect is overwhelmed by the anti-apoptotic activity of synaptic NR2A-containing counterparts. Therefore, our study indicated that at synapses, activation of NR2A- and NR2B-containing receptors have opposing roles in promoting cell survival and death, respectively.

3c


4 h
Difference in Apoptosis
(% relative to control)
50
40
30
20
10
0
Control
NVP
Ro
NVP+Ro

3d

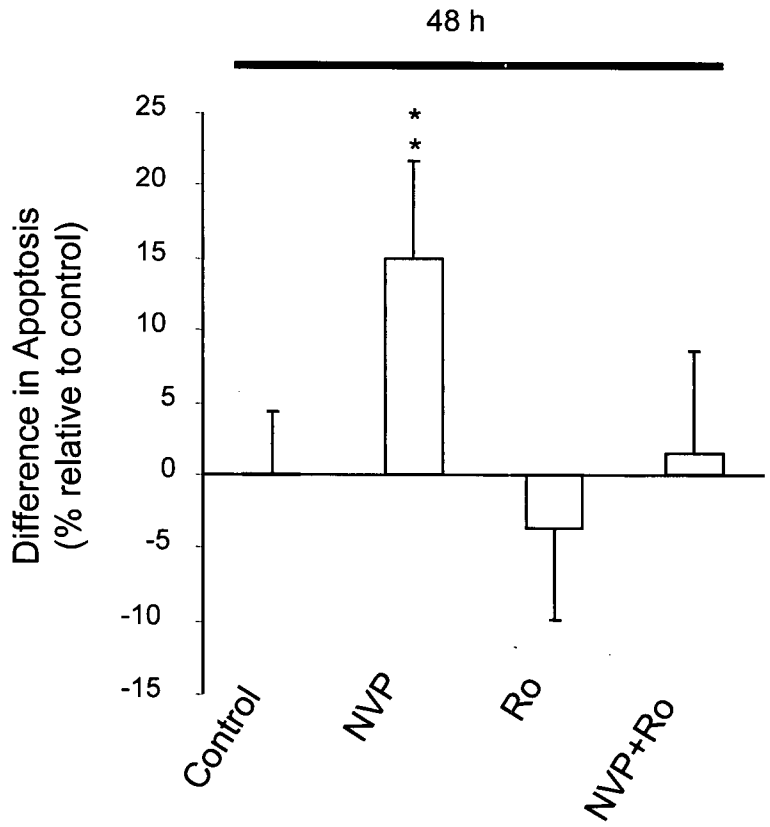

48 h
Difference in Apoptosis
(% relative to control)
25
20
15
10
5
0
-5
-10
-15
**
Control
NVP
Ro
NVP+Ro

Fig. 3c-d. **Spontaneously activated synaptic NR2A- and NR2B-containing NMDARs have opposing roles in promoting neuronal survival and death, respectively. Although a relatively short incubation of neurons with NR2A-specific antagonist NVP (0.4 μM) or NR2B-specific antagonist Ro (0.5 μM) does not have effects on neuronal fate (c), an extended duration (48 h) of NVP but not Ro incubation (in the absence of bicuculline) was sufficient to produce an increase in apoptosis (d). The NVP-induced apoptosis was prevented by addition of Ro (NVP+Ro). These results indicated that both synaptic NR2A- and NR2B-containing subpopulations of NMDARs are spontaneously activated by presynaptically released glutamate, exerting counteracting effects on cell survival and death, but synaptic NR2A-containing receptor activation is predominant and required for maintaining normal neuronal survival. ** $p < 0.01$ compared with control; n=12-18 and n = 15-17 for each group from three separate experiments in Fig. 3c and 3d, respectively.**

3.1.4.2. Extrasynaptically located NR2A- and NR2B-containing NMDARs also have disparate roles in neuronal fate.

3.1.4.2.1. NR2A-containing NMDARs exist at extrasynaptic sites albeit the predominance of NMDARs containing NR2B.

In contrast to the predominant expression of NR2A-containing receptors at synapses, NR2B-containing receptors are thought to be primarily expressed at extrasynaptic sites in mature neurons (Massey et al., 2004b;Tovar and Westbrook, 1999). To determine if some of NR2A-containing NMDARs are also expressed at extrasynaptic sites in the neurons under study, we first pharmacologically blocked all NMDARs expressed at synapses and then examined if currents specifically gated through extrasynaptic NMDARs are sensitive to NR2A subunit-specific antagonist. The selective blockade of synaptic NMDARs was achieved by co-application of bicuculline (50 μM) and MK-801 for 10min. As described above, bicuculline enhances synaptic release of glutamate and thereby selectively activates synaptic NMDARs. MK801, as an irreversible blocker of open NMDAR channels (Huettner and Bean, 1988), only blocks bicuculline-activated synaptic NMDARs, and does not block extrasynaptic channels that are not activated during bicuculline stimulation. The complete blockade of synaptic NMDARs by co-application of bicuculline and MK-801 was achieved within 10 min of drug applications as indicated by the virtual elimination of the slow and APV-sensitive component of mEPSCs (Fig. 4a-1 and 2). Different from what was observed by Tovar et al. (Tovar and Westbrook, 2002), we found little recovery of mEPSCs within one hour following wash out of the drugs. The currents gated through extrasynaptic NMDARs were then induced by fast perfusion of NMDA (200μM) to the MK801 pretreated cells immediately after

washing out bicuculline and MK-801. The extrasynaptic NMDAR-mediated currents could be largely reduced by NR2B antagonist Ro (0.5 μM; Fig. 4a-3 and 4), consistent with the idea that predominant extrasynaptic NMDARs are NR2B-containing (Stocca and Vicini, 1998;Tovar and Westbrook, 1999). The small component of extracellular NMDAR-mediated currents that were resistant with NR2B antagonist were almost completely abolished by NR2A antagonist NVP (0.4 μM; Fig. 4a-3 and 4), indicating the non-NR2B-containing extrasynaptic NMDARs are exclusively NR2A-containing receptors. On average, about 26.6 ± 2.3% (n=5) of total currents gated by extrasynaptic NMDARs was mediated by NR2A-containing receptors (Fig. 4a-4).

Another experiment designed to confirm the results obtained involved reversing the order of NVP and Ro applications as shown in Fig. 4a-3, and then determining the percentage of extrasynaptic NR2A-containing NMDARS. However, since the respective concentrations of NVP and Ro used in the present study show little cross-receptor blockage (Fig. 1a and b), this experiment might be redundant.

Overall, these results provided further evidence for the existence of functional NR2A-containing NMDARs at extrasynpatic sties in the mature cultured cortical neurons.

4a

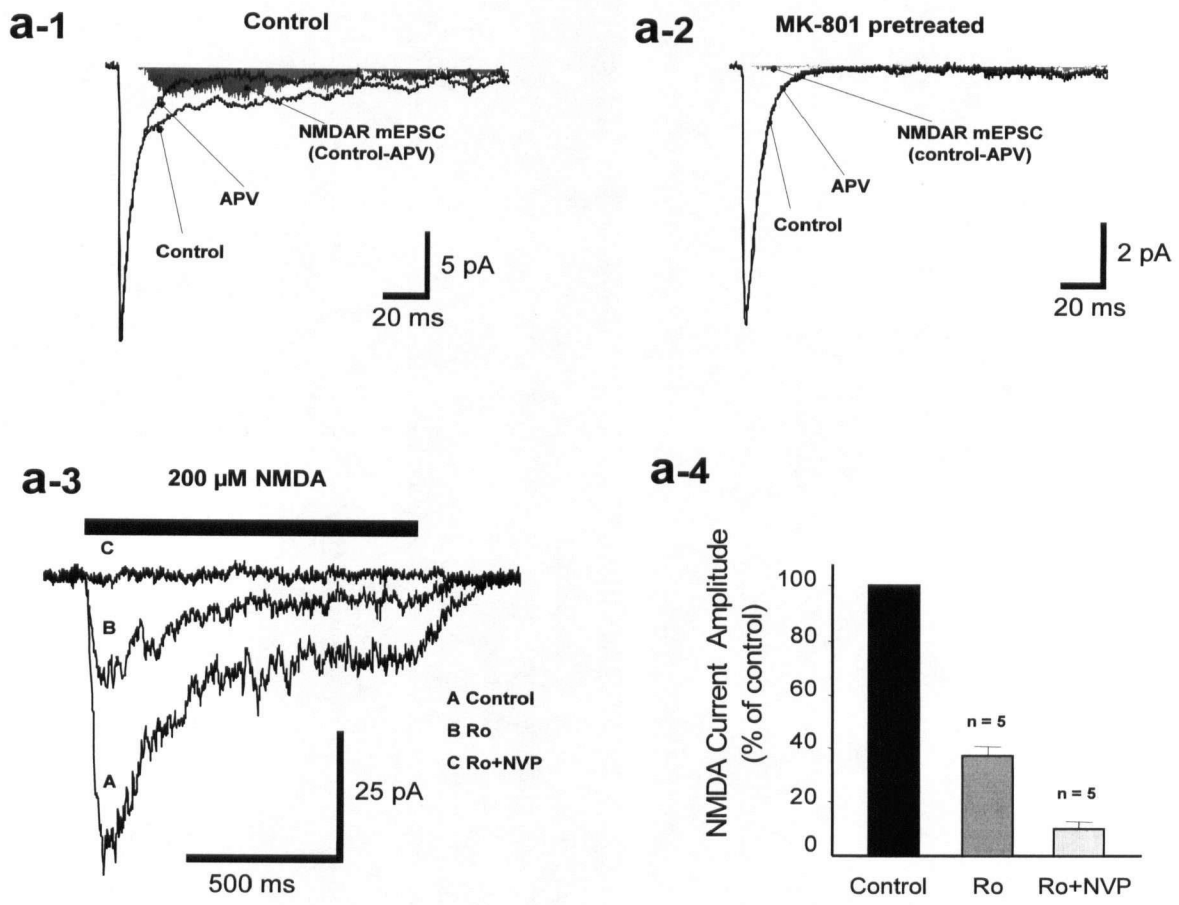

a-1
Control
NMDAR mEPSC
(Control-APV)
APV
Control
5 pA
20 ms
a-2
MK-801 pretreated
NMDAR mEPSC
(control-APV)
APV
Control
2 pA
20 ms
a-3
200 μM NMDA
C
B
A
A Control
B Ro
C Ro+NVP
25 pA
500 ms
a-4
NMDA Current Amplitude
(% of control)
100
80
60
40
20
0
n = 5
n = 5
Control
Ro
Ro+NVP

Fig. 4a. Functional NR2A-containing NMDARs are present at extrasynaptic sites. Whole-cell recordings were performed at a holding membrane potential of -60 mV. a-1. Averaged traces of mEPSCs showing an APV-sensitive (50 μM) NMDAR-mediated component (Control-APV). a-2. Averaged traces of mEPSCs showing the blockade of synaptic NMDARs by the open channel blocker MK-801 (10 μM plus 50 μM bicuculline, 10 min), as demonstrated by the elimination of the NMDAR-mediated component of the mEPSCs (Control-APV). a-3. Example traces of whole-cell currents evoked by NMDA (200 μM) following the blockade of synaptic NMDARs with MK-801 in the absence (control; A) or presence of Ro (0.5 μM; B) or Ro and NVP (0.4 μM; Ro+NVP; C). NMDAR-mediated currents were evoked by fast application of NMDA within 10 min of washing out MK-801 and bicuculline. Currents remaining following the blockade of extrasynaptic NR2B-containing receptors were virtually abolished by the addition of NVP, suggesting the presence of functional extrasynaptic NR2A-containing NMDARs in these neurons. a-4. Histogram shows the summarized data from 5 individual neurons.

3.1.4.2.2. Extrasynaptic NMDARs containing NR2A have pro-survival action which can protect neurons against NMDAR- and non-NMDAR–dependent apoptosis.

Since we found that functional NR2A-containing NMDARs are localized to extrasynaptic sites, the next question we wished to address was the role of extrasynaptic NR2A- and NR2B-containing receptors in mediating NMDA-induced cell survival and death. After specific blockade of synaptic NMDARs and washing out bicuculline and MK-801, the neurons were treated with NMDA (50 μM) for 20min in the absence or presence of NVP (0.4 μM) or Ro (0.5 μM). Quantitative neuronal apoptosis assays performed 20h after the treatments showed that NMDA application alone (non-selective activation of extrasynaptic NMDARs) elicited significant apoptosis ($p<0.001$, Fig. 4b), which could be prevented by selective blockade of NR2B-containing extrasynaptic NMDARs with Ro. In sharp contrast, selective blockade of NR2A-containing receptors with NVP potentiated NMDA-mediated apoptosis (Fig. 4b, $p<0.05$, compared with NMDA). Thus, like synaptic NMDARs, activation of extrasynaptic NR2A-containing receptors has a role in promoting neuronal survival that can counteract NR2B-mediated neuronal apoptosis.

Taken together, the data illustrated in Fig. 3 and 4 strongly indicate that, regardless of their anatomical (synaptic versus extrasynaptic) locations, NR2A- and NR2B-containing receptors have opposing roles in mediating NMDA-elicited neuronal survival and apoptosis and that these opposing roles are dictated by their subunit compositions, but not their anatomical localizations.

4b

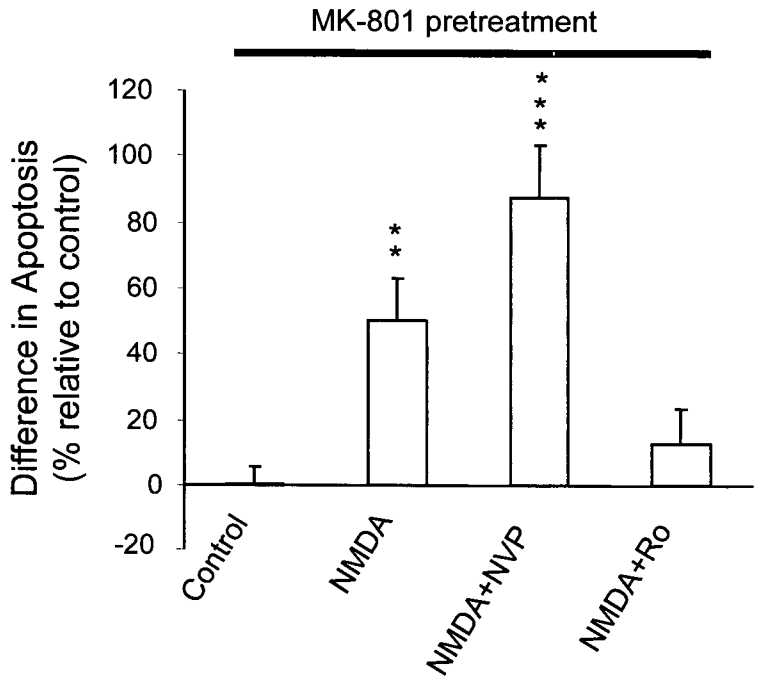

MK-801 pretreatment
Difference in Apoptosis
(% relative to control)
120
100
80
60
40
20
0
-20
***
**
Control
NMDA
NMDA+NVP
NMDA+Ro

Fig. 4b. **Activation of extrasynaptic NR2A-containing NMDARs protects against neuronal death mediated by extrasynaptic NR2B-containing NMDARs. Excitotoxic neuronal death was induced in cortical neurons by bath application of NMDA (50 μM, 20 min) after the blockade of synaptic NMDARs with MK-801 plus bicuculline, and cell death was assayed 20 h later. NMDA elicited neuronal apoptosis, which was exacerbated when the NR2B-containing component was selectively stimulated (NVP+NMDA), but eradicated when the NR2A-containing component was specifically activated (Ro+NMDA). ** $p < 0.01$, *** $p<0.001$ compared with control. n = 11-12 from two separate experiments for each group.**

The finding that NR2A exerts a neuronal survival effect that counteracts NR2B-mediated neuronal apoptosis prompted us to further examine whether the NR2A-mediated neuronal survival effect may also be protective against neuronal damage caused by factors other than NMDAR-mediated excitotoxicity. To selectively stimulate extrasynaptic NR2A-containing receptors with bath application of NMDA, I first irreversibly blocked all synaptic NMDARs with co-application of bicuculline and MK-801 as described previously and then the extrasynaptic NR2B-containing receptors by incubating the neurons in the presence of Ro (0.5 μM) throughout the entire course of the experiments. Under these conditions, bath application of NMDA alone did not increase neuronal apoptosis, confirming the effective blockade of NR2B-mediated pro-apoptotic actions. NMDA-independent neuronal death was then induced by incubation of the cultured neurons with staurosporine (STS), a potent apoptosis inducer (Budd et al., 2000). As shown in Fig. 4c, treatment with STS (100 nM, 1h) alone triggered tremendous neuronal apoptosis. The STS-induced apoptosis was significantly reduced by a brief bath application of NMDA (200 μM; 5 min) prior to STS treatment ($p<0.001$, compared with STS alone, Fig. 4c). This neuronal protection provided by NMDA pretreatment is mediated through extrasynaptic NR2A receptors as it was prevented by co-application of NVP (0.4 μM; $p<0.001$, compared with STS alone).

Therefore, extrasynaptic NR2A-containing NMDARs indeed possess neuronal survival-promoting capability that, once activated, is not only against NMDAR-dependent (NR2B-mediated apoptosis), but also NMDAR-independent neuronal damage.

4c

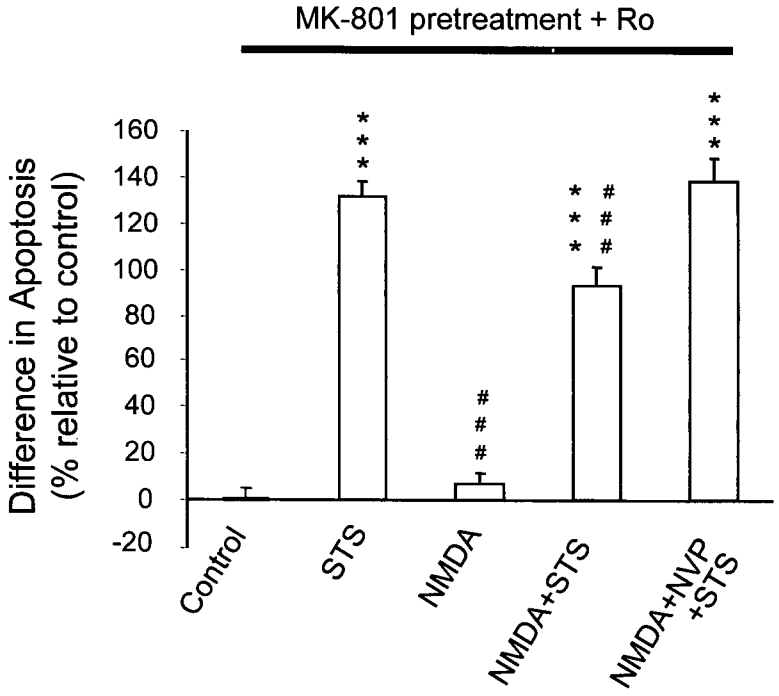

MK-801 pretreatment + Ro
Difference in Apoptosis
(% relative to control)
160
140
120
100
80
60
40
20
0
-20
Control
STS
NMDA
NMDA+STS
NMDA+NVP
+STS

Fig. 4c. **Activation of extrasynaptic NR2A-containing NMDARs can counteract NMDAR-independent apoptosis. Bath application of staurosporine (STS, 100 nM, 1 h), after blockade of synaptic NMDARs with pretreatment of MK-801 plus bicuculline and of extrasynaptic NR2B receptors in the presence of Ro (0.5 μM), induced significant increase in neuronal apoptosis (Ro+STS). Brief application of NMDA (200 μM, 5 min) did not produce neuronal aopotosis on its own (Ro+NMDA), but significantly reduced the STS-induced neuronal apoptosis (Ro+NMDA+STS) and the NMDA-induced neuroprotective action was abrogated by co-application of NVP (0.4 μM; Ro+NVP+NMDA+STS). *** $p < 0.001$ compared with Ro treatment. ### $p < 0.001$ compared with Ro+STS treatment. n = 8-12 for each group from three separate experiments.**

CHAPTER 3: RESULTS

Part II: Treatment of stroke by selective activation of NR2A-containing NMDARs

3.2.1. Summary

Since NR2A-containing NMDARs has anti-apoptotic activity that can protect neurons against NMDAR- and non-NMDAR-dependent apoptosis, in this study I investigated the effects of selective activation of NR2A-containing NMDARs on hypoxic/ischemic neuronal death *in vitro* (OGD) and *in vivo* (MCAo). The data showed that, although pretreatment with NR2B-specific NMDAR antagonist can protect against ischemic injury, post-ischemic inhibition of NR2B has a narrow therapeutic window (ineffective when administered 4.5 h after stroke onset). In striking contrast, specific stimulation of NR2A-containing NMDARs can remarkably reduce ischemic brain damage when carried out either prior to or, most importantly, 4.5 h following ischemic insults. Therefore, treating stroke by selectively activating NR2A-containing NMDARs is a strategy fundamentally different from general NMDAR antagonism, and may represent a novel therapy for stroke.

3.2.2. Selective activation of NR2A-containing reduces hypoxic neuronal injury *in vitro*

The results shown in my previous experiments clearly indicate that activation of NR2A-containing NMDARs may play a critical role in protecting against neuronal damage during brain insults such as stroke in which both NMDA- and non-NMDA dependent neuronal injuries have been implicated. Therefore, I reasoned that selective activation

of NR2A-containing NMDARs may represent a novel therapy for stroke that is completely different from general NMDAR antagonism.

To investigate whether activation of NR2A-containing NMDARs can reduce brain damage resulting from ischemic insults, I first compared the effects of selective blockade of NR2A- and NR2B-containing NMDARs on neuronal apoptosis in a well characterized *in vitro* stroke model, OGD (Goldberg and Choi, 1993). Mature cortical cultures of 11-14 DIV were subject to an anaerobic atmosphere for 1 hour in a glucose-free solution in the absence or the presence of either NVP (0.4 μM) or Ro (0.5 μM). Neuronal apoptosis was quantitatively determined 20 h after OGD. As shown in Fig. 5a, 1 h OGD was able to produce a pronounced increase in neuronal apoptosis. As I expected, selective inhibition of NR2A-containing NMDARs with NVP significantly enhanced OGD-induced neuronal apoptosis ($p<0.05$, compared with OGD), but in contrast, specific blockade of the NR2B-containing NMDARs by Ro markedly attenuated neuronal apoptosis ($p<0.001$, compared with OGD, Fig. 5a). As discussed previously, blockade of NR2A-containing NMDARs virtually results in activation of the NR2B-containing counterparts in these mature cortical cultures, and vice versa; therefore, the data suggested that stimulation of the pro-survival action of NR2A does offer neuroprotection during stroke in vitro.

5a

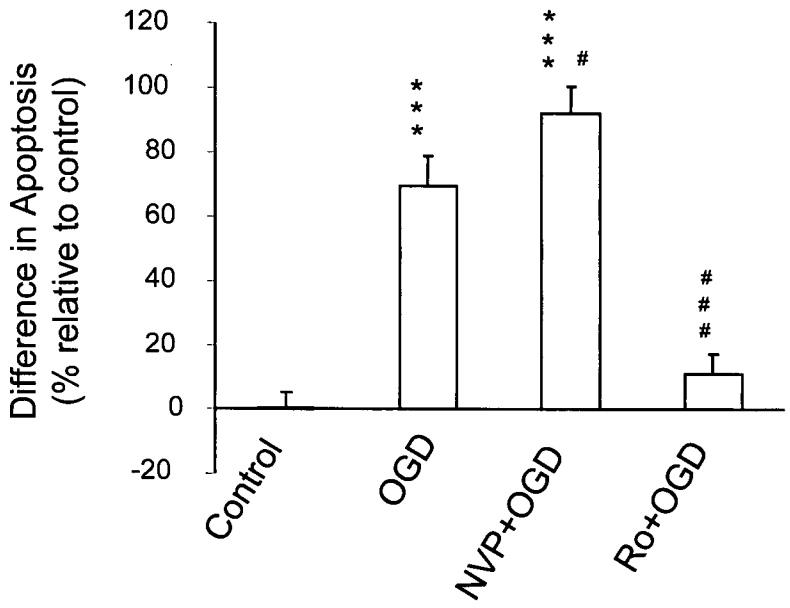

Difference in Apoptosis
(% relative to control)
120
100
80
60
40
20
0
-20
***
***
#
###
Control
OGD
NVP+OGD
Ro+OGD

Fig. 5a. **Pretreatments with NR2A- and NR2B-specific antagonists respectively promote neuronal survival and death in stroke *in vitro*. Cortical cultures were challenged with a 1-h OGD and apoptosis was assayed 23 h after the challenge. OGD resulted in a significant increase in neuronal apoptosis compared with non-challenged controls (Control) and the OGD-induced apoptosis was potentiated by the NR2A specific antagonist NVP (0.4 μM; NVP+OGD) and inhibited by the NR2B antagonist Ro (0.5 μM; Ro+OGD) when bath applied 10 min prior to, and during, the OGD challenge, indicating NR2A- and NR2B-containing receptors exert opposing effects in anoxic neuronal death *in vitro*. *** $p < 0.001$ compared with control. # $p < 0.05$, ### $p < 0.001$ compared with OGD. $n = 17$-18 for each group from three separate experiments.**

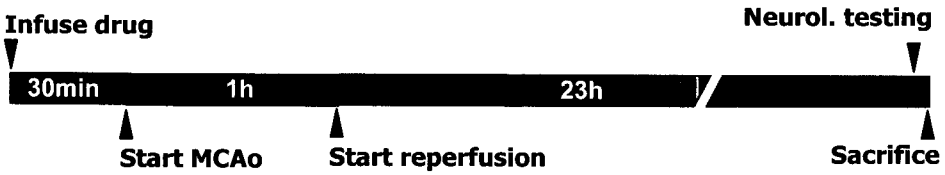
3.2.3. Selective activation of NR2A reduces ischemic brain damage *in vivo*

3.2.3.1. Pretreatment with NR2A-specific antagonist has deleterious effects on ischemic brain damage in rats

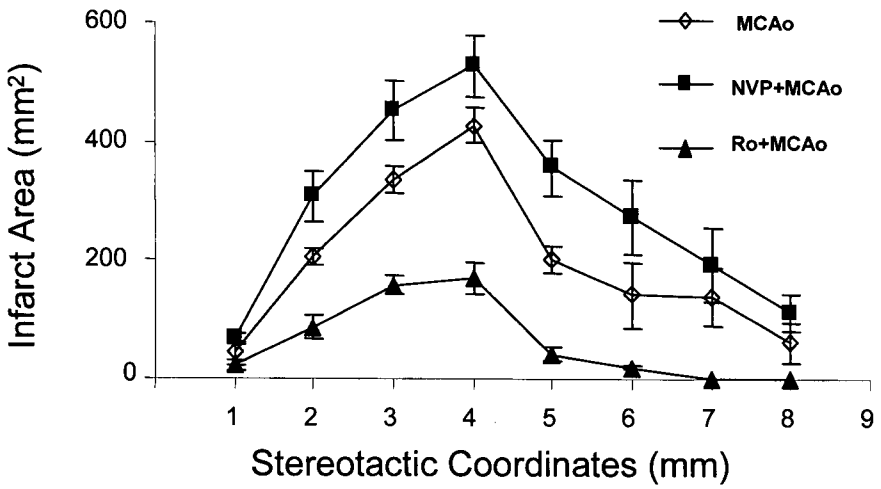
Following the effects observed in the *in vitro* stroke model, I determined whether these findings could be reproduced *in vivo* using a rat focal ischemic stroke model - middle cerebral artery occlusion (MCAo) (Longa et al., 1989). The animals were infused with NR2A specific antagonist NVP (2.4mg/kg, personal communication with Auberson Y.P.) or NR2B specific antagonist Ro (6mg/kg (Loschmann et al., 2004)) or vehicle (saline) through intravenous injection (i.v.) 30 min prior to stroke (MCAo) onset and then subjected to a 1-h transient ischemic stroke induced by MCAo. This relatively short duration of ischemia was chosen to unmask the potential contribution of NR2A-mediated neuroprotective effects. Neurological score and cerebral infarction was examined 24 h after MCAo onset (see Methods and Materials). We found that the infarct areas and the total infarct volume were significantly increased by blockade of NR2A-containing NMDARs through pre-treatment with NVP, but, in sharp contrast, remarkably reduced by NR2B antagonism (Fig. 5b and c). Specifically, when compared with saline-treated animals, NVP pre-treatment gave rise to a 67.0 ± 17.9% increase in total infarct volume ($n=5$; $p<0.05$), while Ro treatment decreased the total infarct volume by 67.8 ± 4.3% ($n=6$; $p<0.01$; Fig. 5b and c). Neurological behavioral test also showed that the NVP-treated animals exhibited a trend toward aggravation while Ro treatment produced a protective effect (Fig. 5d). Taken together, these observations indicate that NR2A- and NR2B-containing NMDAR subtypes play opposing roles in stroke-induced brain damage *in vivo* and blockade of NR2A-containing NMDARs may have negative effects on

excitotoxic brain injury. These findings also suggested that activation of NR2A-containing NMDARs may counteract brain damage resulting from stroke.

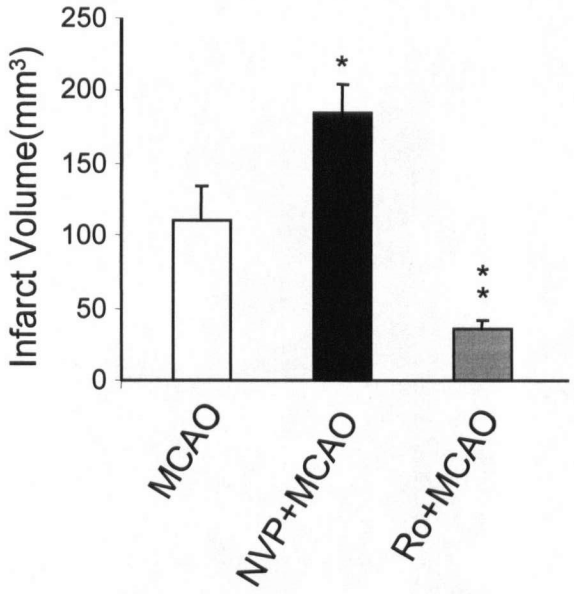
It is noteworthy that the anesthetic isoflurane used in this experiment (and the following *in vivo* experiments as well) may reduce NMDAR-induced excitotoxicity (Harada et al., 1999). To make the results obtained comparable, we anesthetized all the animals in the same manner during the surgery. Therefore, the effects observed should only be attributable to the specific treatment in each experimental group.


Infuse drug
Neurol. testing
30min
1h
23h
Start MCAo
Start reperfusion
Sacrifice

5b


600
400
200
0
Infarct Area (mm²)
1
2
3
4
5
6
7
8
9
Stereotactic Coordinates (mm)
MCAo
NVP+MCAo
Ro+MCAo

5c


Infarct Volume(mm3)
250
200
150
100
50
0
*
**
MCAO
NVP+MCAO
Ro+MCAO

5d

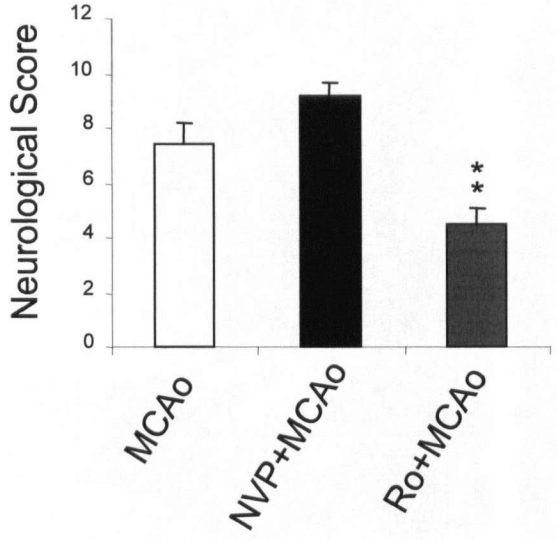

Neurological Score
12
10
8
6
4
2
0
**
MCAo
NVP+MCAo
Ro+MCAo

Fig. 5b-d. **Activation of NR2A- and NR2B-containing receptors exerts opposing effects on ischemic neuronal injuries *in vivo*. Adult rats were subjected to a 1-h focal cerebral ischemia produced by middle cerebral artery occlusion (MCAo), and cerebral infarction was assessed 24 h after MCAo onset. Intravenous infusion 30 min before MCAo onset of NVP (2.4 mg/kg; NVP+MCAo; n = 5) and Ro (6 mg/kg; Ro+MCAo; n = 6) respectively increased and decreased both infarct area (b) and total infarct volume (c). * $p < 0.05$, ** $p < 0.01$ compared with MCAo. d. Neurological scores assessed 24 h after stroke onset in the same groups of animals shown in (b) and (c) indicate that blockade of the NR2A-containing NMDARs resulted in a trend toward worsening neurological function, whereas blockade of NMDARs containing NR2B markedly improved neurological behavior. ** $p < 0.01$ compared with MCAo.**

3.2.3.2. Postischemic potentiation of NR2A-containing NMDARs is neuroprotective

3.2.3.2.1. Glycine can exert neuroprotection through activation of NR2A-containing NMDARs

A practical therapy for stroke would be one that can be implemented following the onset of stroke. Since this study indicates that NR2A-containing NMDAR subtype has a role in promoting neuronal survival, I reasoned that selective activation of such NMDAR subtype after stroke attack would reduce brain damage.

The simplest way to activate NR2A-containing NMDARs specifically is to use a highly selective NR2A agonist. However, to date no NR2A-specific agonists have been identified. To circumvent this, an alternative strategy was designed. The NMDAR co-agonist glycine has been shown previously to potentiate the activation of NMDARs by endogenously released glutamate from presynaptic terminals *in vivo* (De et al., 2000). Moreover, our lab has demonstrated that exogenous application of a suprasaturating concentration of glycine (200 μM), can selectively activate synaptic NMDARs and induce long-term potentiation (LTP) in cultured hippocampal neurons (Lu et al., 2001). Given that NR2A-containing NMDARs are predominant at synapses, and that NR2A-containing NMDARs may be critical for mediation of LTP (Liu et al., 2004a), the effects of glycine on LTP induction must be primarily mediated by potentiation of synaptic NMDARs containing NR2A. Therefore, glycine may be a good agent to enhance activation of NR2A-containing NMDARs and hence may boost neuronal survival. Indeed, our study indicated that, following a brief (10 min) pre-stimulation with glycine (300 μM) and strychnine (10 μM) in Mg^{2+} free ECS, cortical cultures challenged with NMDA (50 μM; 20 min) exhibited significantly less neuronal apoptosis ($p<0.05$,

compare with NMDA alone; Fig. 6a). Strychnine is a highly specific glycine receptor antagonist to which the glycine-binding site on NMDARs is insensitive (Jansen and Dannhardt, 2003). As a competitive antagonist, strychnine at 10 μM is more than sufficient to completely block the activation of glycine receptors by 300 μM glycine (Zhang et al., 2006). Thus, co-application of glycine ensured exclusive enhanced stimulation of the glycine site on NMDARs. The survival-promoting effect of glycine was indeed mediated by activation of the NR2A-containing NMDAR subpopulation at synapses because, in the presence of NR2A-specific antagonist NVP (0.4 μM), glycine pretreatment no longer exhibited neuroprotective effects, whereas in the presence of NR2B-specific antagonist Ro (0.5 μM), glycine still exerted significant neuroprotection ($p<0.01$, compared with NMDA alone; Fig. 6a).

An alternative explanation for the neuroprotective effect of glycine is that pretreatment by glycine leads to ensuing reduction in membrane NMDARs (Nong et al., 2003), and hence NMDAR-mediated excitotoxicity. If the glycine effect was largely due to the decreased membrane NMDAR number, then co-application of NVP, which binds to NR2 but not NR1 (containing glycine binding site), with glycine would not interfere with the neuroprotection by glycine. However, the data shown in Fig. 6a indicated that co-application of NVP virtually eliminated the neuroprotection by glycine pretreatment. Therefore, the endocytosis of NMDARs primed by glycine may contribute minimally to the neuroprotective effect provided by glycine pretreatment in our study.

6a

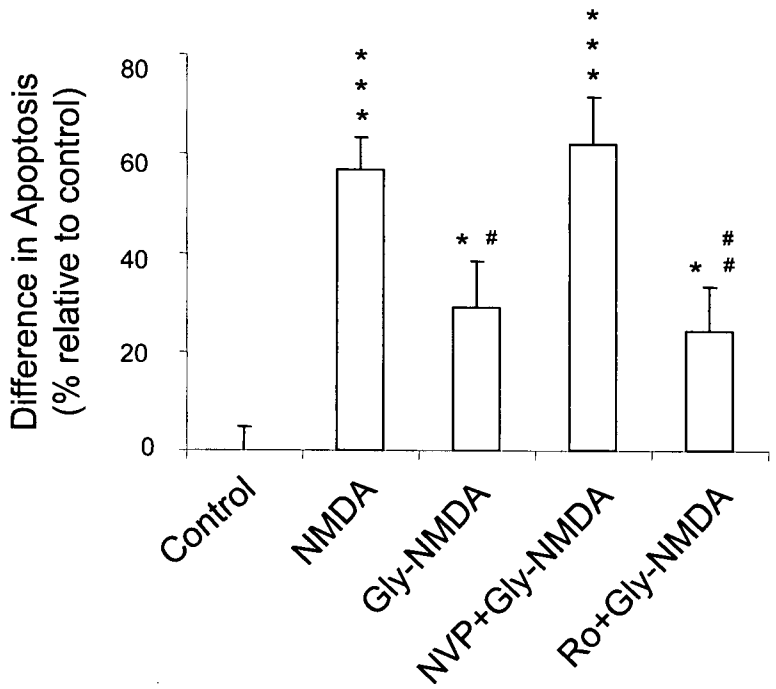

Difference in Apoptosis
(% relative to control)
80
60
40
20
0
Control
NMDA
Gly-NMDA
NVP+Gly-NMDA
Ro+Gly-NMDA

Fig. 6a. **Potentiated activation of NR2A-containing NMDARs by NMDAR co-agonist glycine exerts neuroprotection in vitro. NMDA-induced neuronal apoptosis was significantly reduced by a brief pre-treatment with glycine (300 μM) in the presence of strychnine (10 μM). However, this neuprotective effect was abolished when NR2A-specific antagonist NVP (0.4 μM) was co-applied (NVP+Gly-NMDA), indicating NR2A-containing NMDARs mediated the neuroprotection. In contrast, NR2B-specific antagonist Ro (0.5 μM) was not able to block the effect of glycine. * $p<0.05$, *** $p < 0.001$ compared with control. # $p < 0.05$, ## $p < 0.01$ compared with NMDA. n = 17-18 for each group from three separate experiments.**

3.2.3.2.2. Pretreatment with glycine reduces OGD-induced neuronal apoptosis

After establishing that bath application of glycine can protect neurons from NMDA-induced neuronal death in cortical cultures, I examined if it was neuroprotective in the *in vitro* stroke (OGD) model. Prior to OGD, the neurons were treated briefly (10 min) with glycine (300 μM) in the presence of strychnine (10 μM). Quantification of neuronal apoptosis 20 h following OGD showed that glycine markedly, albeit only partially, decreased OGD-induced cell death ($p<0.001$, compared with OGD; Fig. 6b). Thus, glycine can serve as a neuroprotectant for hypoxia-induced neuronal injury.

6b

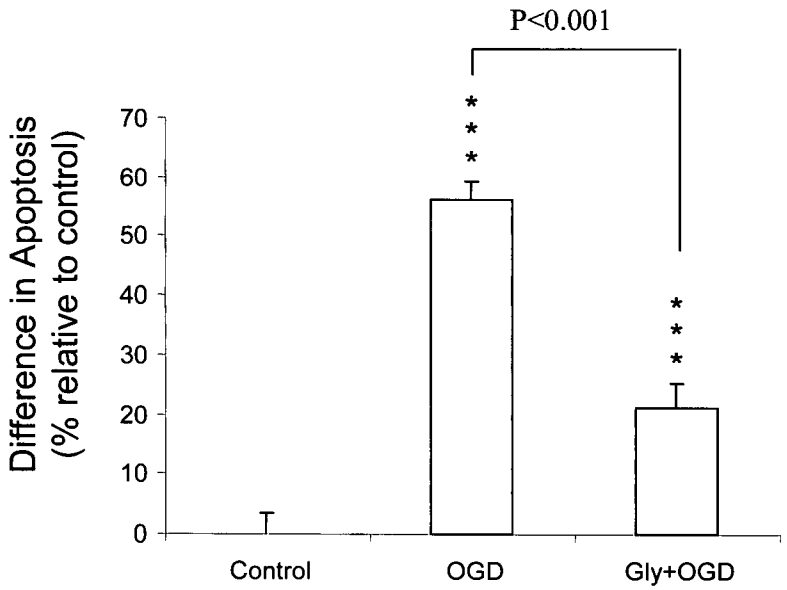

P<0.001
Difference in Apoptosis
(% relative to control)
70
60
50
40
30
20
10
0
Control
OGD
Gly+OGD

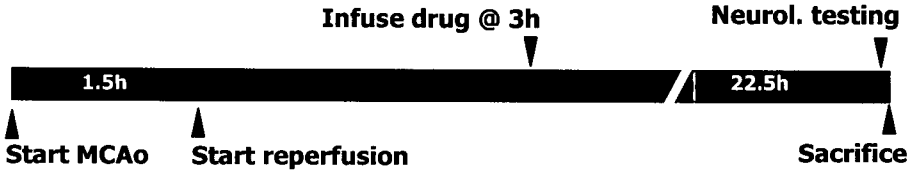
Fig. 6b. Glycine treatment reduces OGD-induced neuronal apoptosis *in vitro*. **Neuronal apoptosis induced by OGD (1 h) was significantly ameliorated by a brief pre-treatment (10 min) with glycine (300 μM) plus strychnine (10 μM), suggesting glycine can exert neuroportection from hypoxic neuronal injury in vitro. *** $p < 0.001$ compared with control. n = 22-24 for each group from four separate experiments.**

3.2.3.2.3. Stimulation of NR2A-containing NMDARs following stroke onset protects the brain from ischemic injury in rats

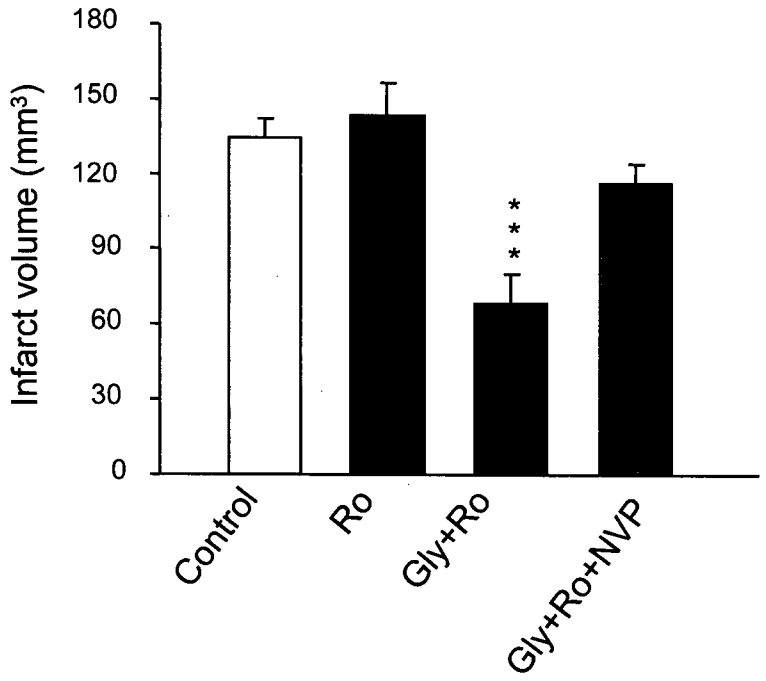
In most clinical situations, a stroke can only be treated after its onset. Thus, a therapy for stroke is not practical unless it can be carried out following stroke onset. Since we have shown selective activation of NR2A is effective in reducing ischemic brain damage before stroke onset, we wanted to determine whether post-ischemic potentiation of NR2A is also neuroprotective.

Given that exogenous application of glycine can primarily activate NR2A-containing NMDARs and exert neuroprotection (Fig. 6a and b), we attempted to use glycine to enhance activation of NR2A-containing NMDARs. Although glycine is an endogenous co-agonist of NMDARs and present in the rat brain at a concentration in the low micromolar range (Danysz and Parsons, 1998), the glycine site on NMDARs may not be saturated in the brain (Danysz and Parsons, 1998;Javitt, 2006;Wood, 1995). Indeed, intraperitoneal injection of a high dose glycine (800 mg/Kg) can enhance the activity of NMDARs *in vivo* (De et al., 2000). Therefore, we attempted to administer glycine to enhance NR2A activation in rats. To exclude the possibility that a large amount of NR2B-containing NMDARs may be simultaneously activated by glycine, NR2B specific antagonist Ro was co-applied during glycine treatment. All the animals were subject to a 90-min transient MCAo. 3 h after reperfusion (4.5 h after stroke onset), the animals were treated with either vehicle (saline) or drug(s) through intraperitoneal injection (i.p.). As presented in Fig. 6c and d, while Ro alone (Ro, 6 mg/Kg, n=10) did not provide neuroprotection when compared with MCAo alone (control, n=10), co-administration of glycine (800mg/kg) with Ro (Gly+Ro, n=9) resulted in remarkable reduction in total

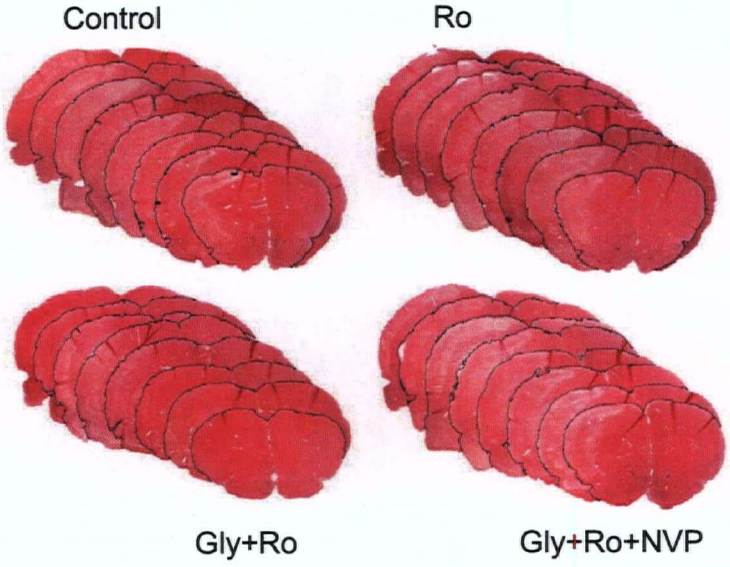
infarct volume assessed 24 h after MCAo onset (49± 8%, $p<0.001$). In line with the lesser infarction, neurological testing 24 h following stroke onset also indicated that glycine improves neurological function ($p<0.001$; Fig. 6e). The efficacy of glycine is mediated through activation of NR2A-containing NMDARs as co-administration of NVP (2.4mg/kg; Gly+Ro+NVP, n=10) was able to reverse both the decrease in infarction and the betterment of neurological behavior to the control (MCAo) levels (Fig. 6c, d and e). These results suggested that post-ischemic potentiation of the pro-survival action of NR2A-containing NMDARs is beneficial for the recovery of brain injury and glycine is a potential neuroprotectant.


Infuse drug @ 3h
Neurol. testing
1.5h
22.5h
Start MCAo
Start reperfusion
Sacrifice

6c


Infarct volume (mm³)
180
150
120
90
60
30
0
***
Control
Ro
Gly+Ro
Gly+Ro+NVP

6d



6e

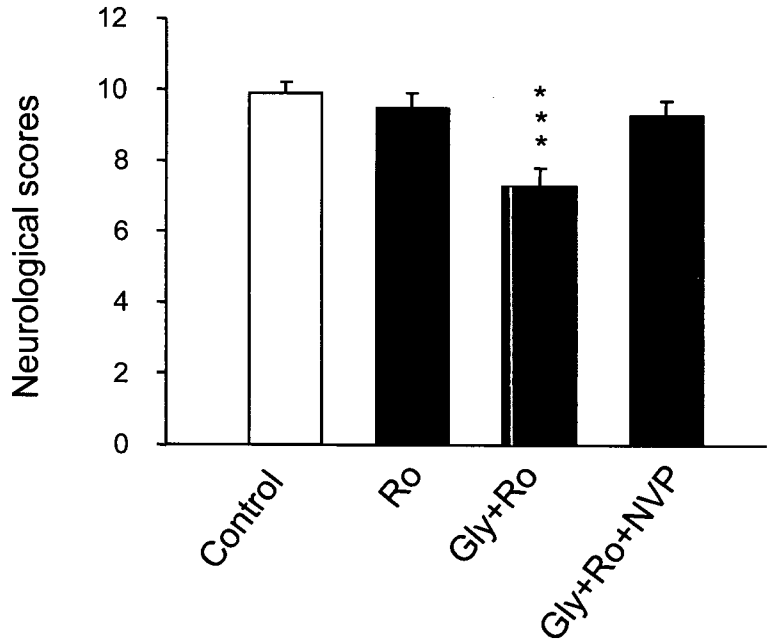


Fig. 6c-e. **Post-ischemic potentiation of NR2A-containing, but not blockade of NR2B-containing NMDARs reduces ischemic brain damage in an *in vivo* focal ischemic stroke model. Adult rats received intraperitoneal injection of either drug or saline 4.5 h after the onset of a 1.5-h MCAo challenge. c. Post-stroke treatment with NR2B antagonist Ro (6 mg/kg; n = 10) was no longer neuroprotective, whereas a post-stroke treatment with NMDAR co-agonist glycine (800 mg/kg) in the presence of Ro (6 mg/kg) significantly reduced total infarct volume (Gly+Ro; n = 9, $p < 0.001$) compared with saline controls (Control; n = 10). The glycine-induced neuroprotection was mediated by enhancement of NR2A-containing receptor activation as it was prevented by co-administration of NR2A antagonist NVP (Gly+Ro+NVP; n = 10). d. Representative brain sections from each treatment group stained with hematoxylin and eosin (H & E). Pale staining indicates infarct. e. Neurological score assessment indicated a significant improvement in neurobehaviour following glycine treatment in the same treatment group. *** $p < 0.001$ compared with Control.**

CHAPTER 3: RESULTS

Part III: Treatment of stroke via disruption of the neurotoxic pathway downstream of activation of NR2B-containing NMDARs

The results presented herein are essentially the same as appeared in: **Michelle Aarts*, Yitao Liu*, Lidong Liu, Shintaro Besshoh, Mark Arundine, James W. Gurd, Yu-Tian Wang, Michael W. Salter, Michael Tymianski (2002). Treatment of ischemic brain damage by perturbing NMDAR- PSD-95 protein interactions. Science 298:846-50** (see Appendix). This project was a collaborative study involving several laboratories of Canadian Stroke Network, but primarily carried out in Dr. Michael Tymianski's lab and ours. I was the primary investigator examining the effects of Tat-NR2B9c peptide on ischemic injury in MCAo stroke model *in vivo*. Due to my contribution to this study, I was assigned one of the two equal first authors.

3.3.1. Summary

Our data indicate that NR2B-containing NMDARs may be the major mediator of excitotoxicity and hence an ideal therapeutic target for stroke. However, the results obtained from *in vivo* study showed that blockade of NR2B has a relatively narrow therapeutic window, and furthermore, blockade of the normal activity of NR2B may lead to untoward side effects. All of these render direct inhibition of NR2B-containing NMDARs an impractical treatment for stroke in clinic. Results from Dr. Tymianski's lab suggested that a post-synaptic density protein PSD-95 specifically couples NMDAR activation to excitotoxicity and that a small peptide derived from the last nine amino

acids of the carboxyal tail of NR2B subunit (KLSSIESDV) can selectively disrupt the interaction between NR2B-containing NMDARs and PSD-95 and reduce NMDA-induced neuronal death *in vitro*. Since perturbing the events downstream of NR2B-containing NMDAR activation may have a broader time window and fewer side effects (due to the avoidance of blocking NR2B-containing receptor functioning), this peptide may be used as an alternative to NR2B blockade to treat stroke. In this study we examined whether this peptide has neuroprotective effects *in vivo*. We found that this peptide significantly attenuates ischemic brain injury when delivered either before or, more importantly, after stroke onset. These findings suggested that perturbing protein-protein interaction downstream of NR2B-containing NMDAR activation may constitute a promising therapy for stroke as well.

3.3.2. Perturbing NR2B-PSD-95 interaction diminishes ischemic brain damage *in vivo*

3.3.2.1. Tat-NR2B9c can be delivered into the brain and perturb the interaction between PSD-95 and NR2B- but not NR2A-containing NMDARs (these experiments were done by Dr. Tymianski's lab)

The NR2B C-tail peptide (NR2B9c) was made cell permeable by fusing to Tat protein (Tat-NR2B9c, See Methods and Materials). Tat-NR2B9c can transduce into cultured cortical neurons readily when bath applied and remain in the neurons for up to 5 h (Appendix, Fig. 1C). To test its feasibility for treatment of ischemic brain injury, they further examined whether this peptide could be delivered into the brain in intact animals when systematically administered (i.p.). C57BL/6 mice (~25 g) were given a 500-μmol

dose of fluorescent peptide Tat-NR2B9c-dansyl or Tat38-48-dansyl (as a cell-impermeant control). Coronal brain sections were taken 1 h after injection and examined by confocal microscopy for fluorescent peptide uptake. They found that the animals injected with Tat-NR2B9c, but not Tat38-48-dansyl, exhibited strong fluorescence in the cortex (Appendix, Supplemental Figure 2A). Similar results were obtained when Tat-NR2B9c-dansyl was infused (i.v.) to rats, confirming that Tat-NR2B9c enters the brain upon peripheral administration.

Next they tested whether NR2B9c could selectively dissociate the interaction between NR2B and PSD-95. The P2 membrane protein fraction of rat forebrain tissue, which is enriched in synaptic structures, was incubated with Tat-NR2B9c or with one of the three controls, Tat38-48, Tat-AA, or Tat-NR2B-AA, each lacking an intact PDZ binding motif (Kornau et al., 1995). Then they co-immunoprecipitated NR2A or NR2B protein with PSD-95 antibody. Western blot analysis showed that Tat-NR2B9c reduced the co-immunoprecipitation of PSD-95 with NR2B but not NR2A (Appendix, Fig. 1D and E). This suggested that the NR2B9c peptide can selectively disrupt the binding of NR2B to PSD-95 and leave the association of NR2A with PSD-95 alone.

3.3.2.2. Pretreatment with Tat-NR2B9c decreases ischemic brain insults

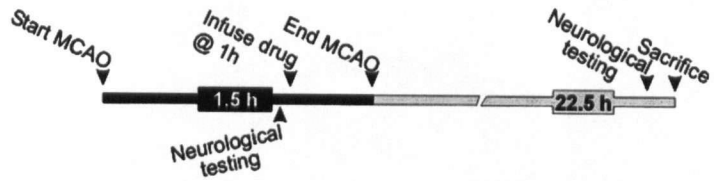
Based on the results from the Tymianski group, Tat-NR2B9c peptide attenuates NMDA-induced excitotoxicity in cultured cortical neurons in vitro. Given that it can be effectively delivered into the brain in the rats and selectively perturb N2B-PSD95 complexes in the brain tissue, we wished to determine whether NR2B9c could be used to treat stroke *in vivo*.

First, we determined whether pretreatment with this peptide effectively in reduced ischemic damage. Rats were pretreated 45 min before stroke (MCAo) by a single bolus injection (i.v.) with saline, the Tat-NR2B-AA control, or Tat-NR2B9c (3 nmol/g; n=6 for each group), and the extent of cerebral infarction was measured 24 h thereafter. Neuronal function testing was performed 60 min during MCAo and 10 min before animals were sacrificed. As shown in Appendix, Supplemental Figure 2C, pretreatment with Tat-NR2B9c reduced the total cerebral infarction volume by 54.6 ± 11.3% ($p<0.01$) was largely accounted for by a 70.7 ± 11.2% reduction ($p<0.01$) in cortical infarction thought to be caused largely by NMDAR-dependent mechanisms. Moreover, this treatment also produced a trend toward improved 24-h neuorological scores (Appendix, Supplemental Figure 2B). In contrast, the control peptide Tat-NR2B-AA had effects on neither neurological function nor cerebral infarction. These results suggested that pre-stroke administration of Tat-NR2B9c ameliorates ensuing brain injury.

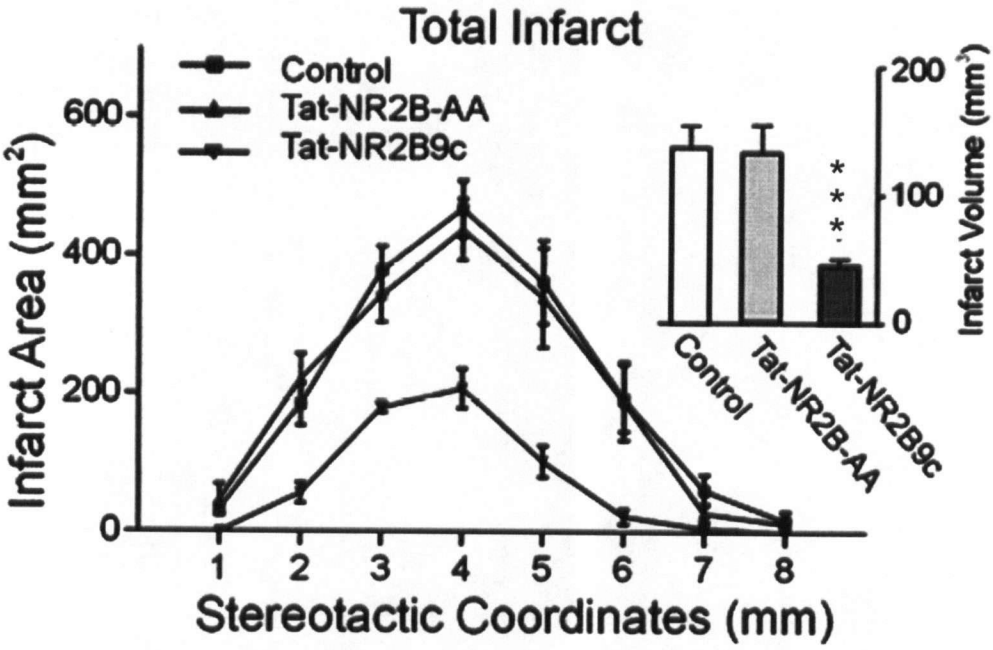
3.3.2.3. Post-treatment with Tat-NR2B9c also attenuates ischemic brain injury and improves neurological function

A stroke therapy would be most therapeutically valueable if effective when given after the onset of ischemia. Thus, we next evaluated whether treatment with Tat-NR2B9c could decrease ischemic damage *in vivo* when applied following stroke onset. Rats were subjected to transient MCAo for 90 min, and saline or Tat peptide (Tat-NR2B9c or Tat-NR2B-AA, 3 nmol/g) was injected (i.v.) 1 h after MCAo onset. Infarction volume and neurological outcome measurements were performed identical to the pretreatment study. Surprisingly, post-ischemic treatment with Tat-NR2B9c caused an even greater reduction

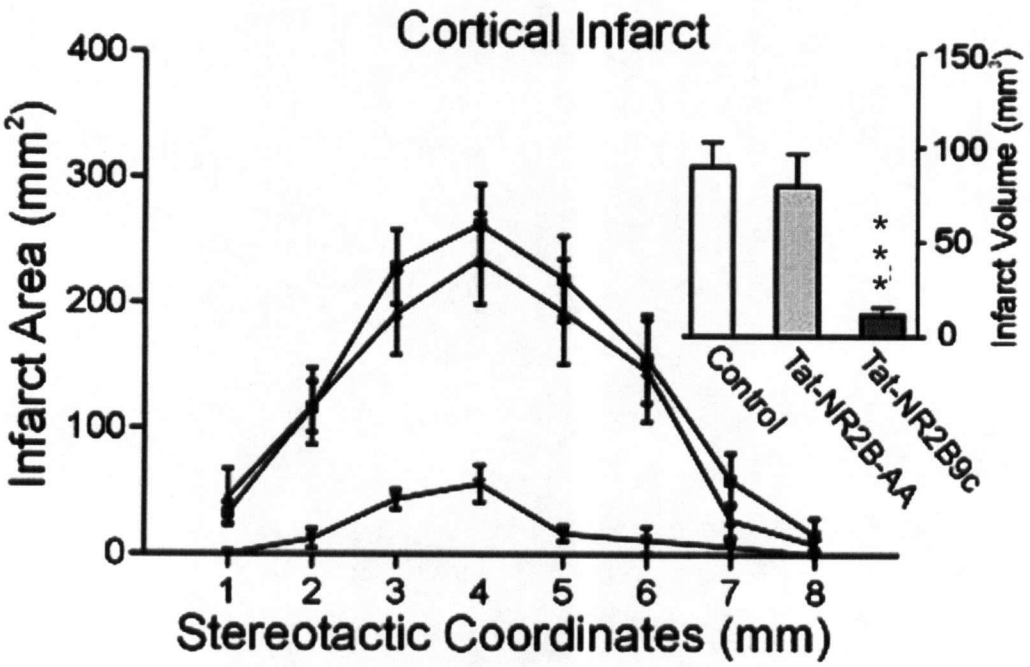
in the volume of total cerebral infarction by 67.0 ± 3.7% (Fig. 7a, $p<0.001$, n=9) when compared with control group (saline, n=10). Similar to the pretreatment study, this reduction was accounted for by an 87.0 ± 4.4% reduction in cortical infarction volume (Fig. 7b, $p<0.001$). These Tat-NR2B9c-treated animals also exhibited a significant improvement in 24-h neurological scores (Fig. 7c, $p<0.001$). In contrast, treatment with Tat-NR2B-AA (n=8) did not have any effects on cerebral infarction or neurological function (Fig. 7c). Thus, Tat-NR2B9c also boosts stroke recovery when given post-ischemically.


Start MCAO
Infuse drug @ 1h
End MCAO
Neurological testing
Sacrifice
1.5 h
22.5 h
Neurological testing

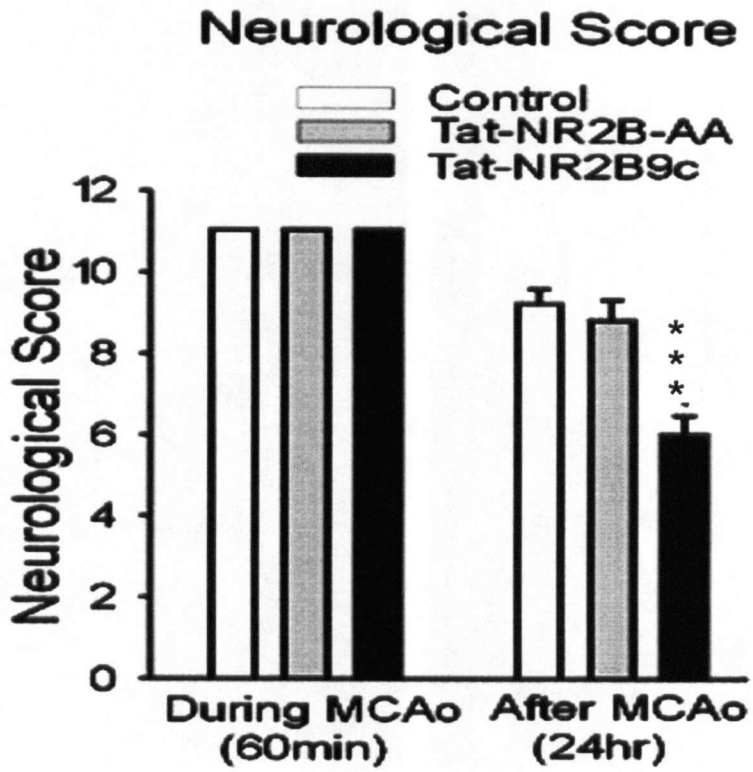
7a


Total Infarct
Control
Tat-NR2B-AA
Tat-NR2B9c
Infarct Area (mm²)
600
400
200
0
1
2
3
4
5
6
7
8
Stereotactic Coordinates (mm)
200
100
0
Infarct Volume (mm³)
***
Control
Tat-NR2B-AA
Tat-NR2B9c

7b


Cortical Infarct
Infarct Area (mm²)
400
300
200
100
0
1
2
3
4
5
6
7
8
Stereotactic Coordinates (mm)
Infarct Volume (mm³)
150
100
50
0
***
Control
Tat-NR2B-AA
Tat-NR2B9c

7c


Neurological Score
Control
Tat-NR2B-AA
Tat-NR2B9c
Neurological Score
12
10
8
6
4
2
0
***
During MCAo
(60min)
After MCAo
(24hr)

7d

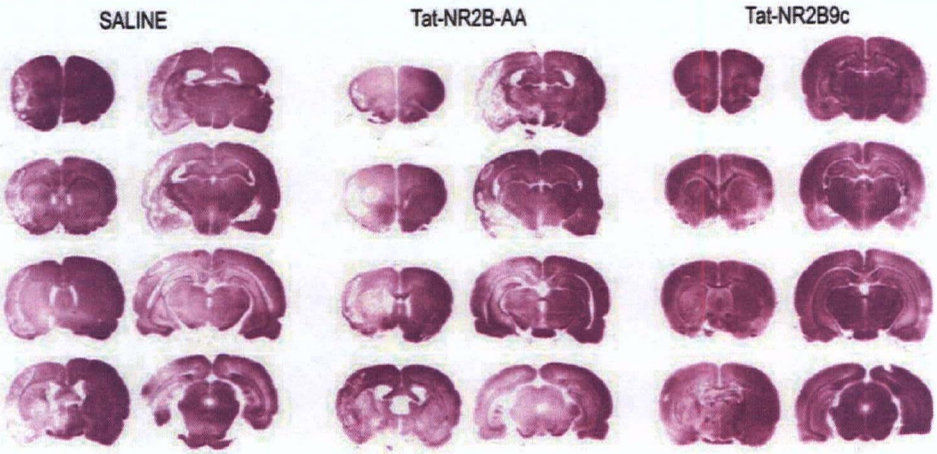


Fig. 7. **Neuroprotection by treatment with Tat-NR2B9c *in vivo*. a, b) Treatment with Tat-NR2B9c (3 nmol/g, n=9) but not mutated Tat-NR2B-AA (n=8) or saline controls (n=10) significantly reduced (a) total infarct area and volume (inset) and (b) cortical infarct area and volume (inset), measured 24 hours after transient MCAO. *** denote $p<0.001$, compared with control. c) Composite neurological scores during and 24 hours after MCAO. *** $p < 0.001$, compared with control. d) Representative appearance of hematoxylin and eosin-stained rat brain sections from each treatment group.**

CHAPTER 4: DISCUSSION

In this thesis research project, the roles of the two major NMDAR subtypes in mature cortical neurons, NR2A- and NR2B-containing NMDARs, in excitotoxicity were investigated. The results showed that, while NR2B-containing NMDARs mediate neuronal apoptosis, the NR2A-containing counterparts have an opposing role—activation of NMDARs containing NR2A can protect against neuronal death. It was also demonstrated that coupling to distinct downstream signaling pathways may underlie the mechanisms by which these two NMDAR subtypes function differentially. Based on these findings, two novel therapies for stroke were designed: the first was to selectively activate the NR2A-containing NMDARs, and the second was to disrupt the signaling pathway downstream of activation of NR2B-containing NMDARs. In *in vitro* and *in vivo* stroke models, both therapies were substantiated to be efficacious in reducing ischemic brain damage even when implemented following stroke onset. Thus, this study provides a molecular basis for the paradoxical roles of NMDARs in promoting neuronal survival and mediating neuronal damage, and as well, at least partially, an explanation for the failure of NMDAR antagonists in previous clinical trials of stroke. Furthermore, the data suggest that NMDAR-based therapies can still be efficacious; however, rather than general NMDAR antagonism, a practical treatment should aim at selective enhancement of NR2A-containing NMDAR activation or perturbing the signaling pathway downstream of activation of NMDARs containing NR2B. These novel therapies may represent a new direction in the treatment of stroke.

4.1. The use of NVP AAM077 as a selective antagonist for NR2A

Auberson et al first showed that NVP has a 130-fold preference for recombinant human NR1/NR2A over NR1/NR2B receptors expressed in oocytes (Auberson et al., 2002). The selectivity of NVP was also confirmed by Liu et al. Using hippocampal cells, these authors found that at a concentration of 0.4 μM, NVP could effectively inhibit NR2A-containing NMDARs while essentially sparing the NR2B-containing subtype (Liu et al., 2004a). On the other hand, Feng et al. found that NVP not only has a poor selectivity for recombinant rodent NMDARs (only 13-fold over NR2B), but also acts as a potent antagonist for NR2C- and NR2D-containing receptors, which suggested, at least in rodents, NVP is not a subunit-specific antagonist whatsoever (Feng et al., 2004). Bererich et al. tested the effects of NVP at the same concentration used in our study (0.4 μM) and found that it blocked around 67% of NR1/NR2B receptors expressed in HEK cells (Berberich et al., 2005a). These studies seem to suggest that NVP may be a poor NR2A specific antagonist. More recently, Weitlauf et al. demonstrated that NVP displays time-dependent blockade kinetics: while simultaneous application of NVP with NMDA does not result in significant inhibition of recombinant NR1/NR2B receptors, pre-appliaction of NVP may produce substantial inhibition of peak currents mediated by these NR2B-containing receptors (Weitlauf et al., 2005b). Although the conflicting results may be partially due to the differential experimental conditions in these studies, the reasons for the distinction remain to be determined.

We found that at the concentrations used in our study, NVP only has a negligible cross-receptor inhibition of NR2B-containing receptors. Under the conditions in our experiments, NVP is sensitive enough to distinguish the roles of NR2A- and NR2B-

containing NMDARs in neuronal fate. Our results clearly indicated that the neuronal survival action can be fully blocked by NVP. Although NVP may have a small contaminant inhibition of NR2B receptors, the lack of blockade of the NMDA receptor-mediated cell survival action by the NR2B antagonist Ro essentially rule out the contribution of this subunit. On the other hand, the efficient blockade of NMDA receptor-dependent cell death by Ro, but not by NVP, strongly suggested that it is the NR2B- but not NR2A-containing NMDA receptor subpopulation that plays a primary role in triggering intracellular cascades that leading to NMDA- or ischemia-induced neuronal apoptosis. Whether NVP blocks other NMDAR subtypes (NR2C- and/or NR2D-containing NMDARs) has not been considered in this study due to their low expression in mature cortical neurons.

4.2. Dual role of NMDARs in neuronal fate and the possible underlying mechanisms

Although it has been well known that the NR2 subunits contribute to the distinct pharmacological and electrophysiological properties of NMDARs, the functional diversity of NMDARs conferred by NR2 subunits remains largely unknown. Compelling evidence seems to suggest that most NMDAR subtypes, such as those containing NR2A-, NR2B- and NR2C subunits, are all involved in mediating excitotoxicity during stroke (Morikawa et al., 1998;Wang and Shuaib, 2005;Kadotani et al., 1998). In this thesis project, we have established for the first time that, as opposed to stimulation of NR2B-containing NMDARs, activation of the NR2A-containing counterparts has pro-survival action in mature cortical cultures and the brain of adult rats. Therefore, NMDAR activation can produce either neuronal survival or death promoting action.

This work has provided a molecular basis to explain the paradoxical roles of NMDAR antagonism in producing extensive neuronal apoptosis in developing animals (Ikonomidou et al., 1999a) and neuroprotection against ischemic brain damage in animal studies (Lee et al., 1999a;Arundine and Tymianski, 2004b). For instance, under normal conditions spontaneously released glutamate preferentially stimulates synaptic NMDARs which comprise mainly NR2A-containing NMDARs, and hence activates predominantly the NR2A-containing receptor-dependent cell survival signaling, thereby playing an essential role in maintaining the physiological survival of the neurons. As demonstrated in this project, NR2A-containing receptor activation, in addition to counteracting NR2B-containing receptor-mediated cell death, has the ability to guard against non-NMDAR-mediated apoptotic processes which may be particularly active as part of the process of eliminating excess numbers of neurons in the developing animal. Thus, blocking these synaptically activated NMDARs may lead to extensive neuronal apoptosis. However, under some pathological conditions, such as stroke and brain trauma, there is usually a transient and rapid increase in extracellular glutamate concentrations, and consequently extrasynaptic receptors, which are predominantly NR2B-containing and not usually activated by synaptically released glutamate under normal synaptic transmission, become activated, resulting in a predominant activation of the NR2B-containing receptor-dependent cell death pathway. Thus, blocking these receptors can be neuroprotective under these pathological conditions.

The pro-survival role of NR2A-containing NMDARs agrees with the observation that a selective reduction in NR2A subunit renders the brain more vulnerable to glutamate excitotoxicity in young animals (Gurd et al., 2002;McDonald and Johnston, 1990). On

the other hand, that NMDAR-dependent excitotoxity is found to be largely attributable to NR2B-containing NMDARs is in accord with previous reports showing that NR2B-selective NMDAR antagonists exhibit excellent neuroprotective effects during stroke (Wang and Shuaib, 2005), and that the CA1 area of hippocampus, which is particularly sensitive to ischemia, expresses greater levels of NR2B subunit (Coultrap et al., 2005). The pro-death activity of NR2B-containing NMDARs is also consistent with that specific disruption of the neurotoxic signaling pathway downstream of NR2B dramatically can attenuate excitotoxicity without affecting NMDAR activity (Aarts et al., 2002). Furthermore, it was demonstrated that in Huntington's disease, striatal neurons selectively damaged via apoptosis show a high density of the NR2B-containing NMDARs, suggesting high levels of NR2B may sensitize these neurons to apoptosis (Li et al., 2003;Zeron et al., 2002).

Interestingly, the amount of cell death induced by selective activation of extrasynaptic NR2B-containing NMDARs (Fig. 4b) was comparable to that shown in Fig. 2b whereby whole cells were stimulated, but much greater than that evoked by specific stimulation of synaptic NMDARs containing NR2B indicated in Fig. 3d. This difference could be due to the different protocols used to stimulate NMDARs in the experiments. We took advantage of the spontaneously released glutamate to examine the role of synaptic NMDARs on neuronal fate. In contrast, exogenous NMDA of 50 μM was bath applied continuously for 20 min to stimulate NMDARs on whole neurons or extrasynaptic sites. Alternatively, the amount of neuronal death may be largely dependent on the amount of activated NR2B-containing receptors. The pro-survival action of NR2A-containing receptors may only counteract the NR2B-mediated neuronal death to a certain extent.

Once the majority of NR2B receptors are activated, the NR2B-mediated death-promoting action will be overwhelming.

The findings presented in our study are seemingly contradictory to those observations using transgenic mice. It has been shown that NR2A-knock-out mice are viable and more resistant to ischemic insults in adulthood (Morikawa et al., 1998), whereas NR2B-knock-out mice die shortly after birth (Kutsuwada et al., 1996). However, knocking out of an NMDAR subunit gene may change the normal subunit composition, distribution and functioning of NMDRs in adulthood. Thus, the data obtained from knockout studies may not reflect the real situations in adult animals that developed normally. Further investigations will be needed to explain these discrepancies.

The mechanisms underlying the distinction of NR2A- and NR2B-containing NMDARs in excitotoxicity remain to be studied. Recent evidence suggests that different NR2 subunits may couple to disparate postsynaptic signaling pathways, most likely via distinct protein interactions with their cytoplasmic carboxyl tails (Sheng and Pak, 2000;Kohr et al., 2003). In the present study we examined the classical Akt cell survival signaling pathways, and found that activation of NR2A-containing NMDARs is specifically coupled to Akt phosphorylation. Why activation of NR2A-containing can selectively lead to Akt activation remains to be determined. It has been shown that stimulation of NR2A-containing NMDARs can preferentially activate Ras-Erk pathway (Kim et al., 2005). Since Ras is also an upstream activator of Akt (Sutton and Chandler, 2002), Akt may be concomitantly and selectively activated upon activation of NR2A-containing NMDARs. Indeed, it has been shown NMDAR activation can trigger both Akt (Sutton and Chandler, 2002) and Erk phosphorylation (Chandler et al., 2001). Recently, a PI3K-dependent pro-

neuronal survival signaling has been linked with NR2A-containing NMDAR activation (Lee et al., 2002), suggesting the PI3K-Akt pathway may be specifically coupled to NR2A activation as well. Here we also investigated the caspase 3-mediated apoptotic pathway. The data suggest that NR2B-dependent neuronal apoptosis may be mediated by activation of caspase 3 and inhibition of Akt phosphorylation. The mechanisms underlying this specificity are unclear, either. However, recent evidence indicates that Akt can be negatively regulated by the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10), a lipid phosphatase that dephosphorylates PI3K. PTEN forms a complex with NR2B- but not NR2A-containing NMDARs *in vivo* (Ning et al., 2004), suggesting activation of NR2B-containing NMDARs may facilitate activation of PTEN, and hence inactivation of Akt. Moreover, SynGAP, a PSD protein that inhibits the activity of Ras, preferentially associates with NR2B-containing NMDARs in the brain, and stimulation of NR2B-containing NMDARs has been reported to specifically inhibit Ras-ERK pathway (Kim et al., 2005). Therefore, through preferential interaction between NR2B and SynGAP, activation of NR2B-containing NMDARs may result in the specific inhibition of Ras, which in turn decreases Akt phosphorylation. Bad is a pro-apoptotic Bcl-2 family protein lying upstream of caspase 3 activation in the intrinsic apoptotic signaling pathway (Wang et al., 1999). However, only when Bad is dephosphorylated and translocated to mitochondria can it lead to activation of caspase 3. Given the critical role of Akt in the phosphorylation of Bad (Datta et al., 1997), NR2B-mediated Akt dephosphorylation may facilitate Bad dephosphorylation, and hence the activation of the caspase-3. Mounting evidence has demonstrated the involvement of caspase-3 in NMDAR-dependent neuronal apoptosis. The results shown here indicate that NMDARs

containing NR2B may be primarily responsible for the activation of caspase-3 in mature cortical neurons. How activation of NR2A- and NR2B-containing receptors initiates distinct signal transduction pathways is largely unknown. The distinct gating kinetics of these two receptor subpopulations (Erreger et al., 2005;Cull-Candy and Leszkiewicz, 2004) may lead to cell-survival or -death signaling by providing differentially required levels and kinetics of rising $[Ca^{2+}]_i$ in the postsynaptic neurons (Lipton and Nicotera, 1998;Choi, 1994). In addition, it has been shown that the N-terminal domains of NR2 subunits may be involved in the regulation of NMDARs (Erreger and Traynelis, 2005;Krupp et al., 1998). Thus, the difference between the N-terminals of NR2A- and NR2B-containing receptors may lead to distinct signaling pathways as well.

Whatever the precise mechanisms are, this study has established that NR2A- and NR2B-containing subpopulations of NMDARs have differential roles in mediating neuronal survival and death, and hence provides a molecular basis for the paradoxical actions of NMDAR antagonism in producing both pro- and anti-apoptotic effects under different conditions.

4.3. Subunit composition of NMDARs determines their role in excitotoxicity

The results from in this study demonstrate that, despite the predominance of NR2A-containing NMDARs, a significant amount of NMDARs containing NR2B is also localized to excitatory synapses, and these NR2B-containing NMDARs are functional. NR2 subunits of NMDARs undergo a diverse temporal and regional distribution change during development. Prenatally, NR2B is predominant, whereas NR2A expression increases quickly after birth (Monyer et al., 1994). In developing neurons, NR2A-

containing NMDARs are inserted into the synaptic sites and replace the existent synaptic NR2B-containing NMDARs with the formation of excitatory synapses, rendering NR2A-containing NMDARs the predominant NMDAR subtype at glutamatergic synapses (Tovar and Westbrook, 1999). Intriguingly, it is also argued that the switch in synaptic NMDAR subunit compostion is not due to the displacement of NR2B by NR2A; instead, the predominance of NR2A-containing NMDARs at synapses may reflect the formation of new synapses from which NR2B is lacking (Liu et al., 2004c). NMDARs can move laterally on the cell surface from extrasynaptic to synaptic localization (Groc et al., 2004), but it remains unclear whether the NR2A-containing NMDARs inserted into synapses are from extrasynaptic sites or directly from the NR2A pool in the cytosol. It is commonly believed that NR2B-containing NMDARs accounts for a small fraction at synapses. This may be due to the more robust endocytosis of NR2B (Lavezzari et al., 2004) and/ or the constant removal of NR2B from synapses even when there is no synaptic activity (Barria and Malinow, 2002). However, our results demonstrated that in the majority of mature cortical neurons, functional NR2B-containing NMDARs make up around one third of synaptic NMDARs. Our findings may provide new insights into the NMDAR subunit composition at excitatory synapses.

It was also found that at extrasynaptic sites, functional NR2A-containing NMDARs, albeit a small portion, co-localize with their NR2B-containing counterparts, which are generally considered to be predominant at these sites at least in mature cortical and hippocampal neurons. Similar to synaptic NMDARs, the NR2A component has been shown to increasingly contribute to extrasynaptic NMDARs during development although the NR2B component is still predominant (Mohrmann et al., 2000). Thus,

extrasynatpic receptors seem to undergo a change in subunit composition as well. Interestingly, NMDARs containing NR2A with truncated C-tail are located at extrasynaptic but not synaptic sites (Steigerwald et al., 2000), suggesting the localization of extrasynaptic NR2A-containing NMDARs does not require tethering proteins interacting with NR2A C-tail.

In contrast with previous data, a recent study suggested that NR2A- and NR2B-containing NMDARs might be evenly distributed at synaptic and extrasynaptic sites in hippocampal neurons (Thomas et al., 2006). Further evidence will be needed to confirm these findings. It is also noteworthy that triheteromeric NMDARs which contain NR1 and two different types of NR2 subunit (Cull-Candy et al., 2001;Sheng et al., 1994), e.g., NR1/NR2A/NR2B may also exist in cortical neurons. Using co-immunoprecipitation method, Luo et al. even found that most NMDARs in the cortex in rats contain both NR2A and NR2B. Data from Tovar et al. also suggested that at hippocampal synapses, NR1/NR2A/NR2B receptors are predominant (Tovar and Westbrook, 1999). However, the portion of these triheteromers in the brain is controversial (Blahos and Wenthold, 1996;Liu et al., 2004c). Whether these triheteromeric receptors possess the properties of both NR1/NR2A and NR1/NR2B receptors is largely unknown. Hatton and Paoletti showed that NMDARs containing a single copy of NR2A or NR2B are still sensitive to subunit-specific agents (zinc or ifenprodil) although the maximal level of inhibition was greatly reduced when compared with those diheteromers containing two copies of NR2A or NR2B (Hatton and Paoletti, 2005). Due to the unknown properties of these triheteromeric receptors, their contribution to neuronal survival or death remains to be investigated.

Although NMDARs are localized to both synaptic and extrasynaptic sites, I have shown here that the dual action of NMDARs on neuronal fate is dictated by their subunit composition and not subcellular (synaptic vs. extrasynaptic) localization. Activation of NR2B-containing NMDARs, synaptic or extrasynaptic, initiates apoptotic signaling cascades and promotes neuronal death. Conversely, selective activation of NR2A-containing NMDARs stimulates pro-survival signaling, thereby exerting a neuroprotective action against both NMDAR- and non-NMDAR-dependent neuronal injuries. Thus, the net impact of NMDAR activation on neuronal survival and death is dictated by the balance between the activation of NR2A- and NR2B-containing NMDAR subpopulations but not by the subcellular locations. However, because of the preferential expression of the two subunits at synaptic versus extrasynaptic sites, selective activation of synaptic and extrasynaptic NMDARs could be expected to respectively result in the activation of predominantly NR2A-containing receptor-dependent cell survival and NR2B-containing receptor-mediated apoptotic pathways. Thus, our results may not be in conflict with recently reported differential actions on cell survival and death following selective activation of synaptic and extrasynaptic NMDARs (Hardingham et al., 2002). Furthermore, most recently it has been shown that synaptic NMDARs can mediate neuronal death as well, confirming that the subcellular location of NMDARs does not decide the role of NMDARs in excitotoxicity.

4.4. Selective activation of NR2A-containing NMDARs represents a novel therapy for stroke

Current strategies for stroke treatment fall into two categories: thrombolysis and

neuroprotection. Despite extensive research, only one thrombolytic, t-PA, has been successful in the treatment of acute stroke (NINDS, 1995). However, the efficacy of t-PA is still controversial (Hacke et al., 1998;Albers et al., 2002). Although t-PA may effectively reduce stroke-induced brain injury, it must be administered within 3-6 h after the onset of ischemic stroke. It is estimated that in the US only no more than 7% patients can receive this therapy. While a many neuroprotective agents have been studied, the future of neuroprotection is even more dismal. To date none of the neuroprotectants tested clinically have proved to be effective, including a variety of NMDAR antagonists (Lutsep and Clark, 2001).

The data shown here indicate that selective activation of NR2A-containing NMDARs can effectively ameliorate ischemic brain damage. Treatment of stroke by specifically activating NR2A-containing NMDARs may have several obvious advantages over previously proposed NMDAR antagonism-based stroke therapies: first, as demonstrated in the present work, it possesses a broader therapeutic window than NR2B-containing receptor blockade. In fact, theoretically it should not have any therapeutic window limitation as it protects neurons against brain damage by promoting neuronal survival rather than blocking the activation of a death signal initiated by the stroke insult; second, it is significant to point out that NR2A-containing receptor activation is not only effective against NMDAR-mediated cell death (primary neuronal injuries), but can also guard against non-NMDAR-mediated cell death (secondary neuronal injuries). Increasing evidence supports the fact that some of the non-NMDAR-mediated mechanisms, while secondary to NMDAR activation, may contribute significantly to brain damage, particularly following severe stroke insults (Xiong et al., 2004;Aarts et al., 2003a). Thus,

activation of NR2A-containing receptors, rather than blockade of NMDARs, appears to be a more effective post-stroke neuroprotective therapy. In addition to the neuronal injuries caused by acute brain insults such as stroke and brain trauma, activation of NR2A-containing receptor-dependent pro-survival signaling may also prove to be a potential neuroprotective therapy for a number of chronic neurodegenerative disorders in which a "slow" NMDAR-mediated excitotoxicity is implicated, such as Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and Alzheimer's disease (Ikonomidou and Turski, 2002b;Lipton and Rosenberg, 1994).

According to the observations in this study, an NR2A-containing receptor activation-based neuroprotective strategy that can diminish both acute brain injury following stroke or brain trauma and chronic brain damage associated with a large number of neurodegenerative diseases may represent a novel therapy for these diseases. Thus, this work calls for the urgent development of highly specific agonists for the NR2A-containing NMDAR subtype. In the absence of such subunit specific agonists, a specific enhancement of NR2A-containing NMDAR function, as demonstrated in the present work, may be achieved by the combination of a non-subunit specific NMDAR enhancer, such as glycine, and an NR2B specific antagonist. Glycine is an endogenous co-agonist of NMDARs. Probably because of its relatively high concentration in the brain under physiological conditions (Danysz and Parsons, 1998), it is commonly assumed that glycine is freely available for NMDARs and the glycine site on NMDARs is always saturated. However, recent evidence suggests that the glycine site may be unsaturated *in vivo* (Wood, 1995); (Javitt, 2006;Danysz and Parsons, 1998). This may explain why in our study the activity of NMDARs, NR2A-containing receptors in particular, can be

enhanced by a high dose of exogenous glycine. It is interesting to note, in this regard, that both NMDAR glycine site agonists, such as D-cycloserine (Posey et al., 2004), and NR2B specific antagonists(Chazot, 2000) are now available for clinical trials. Thus, our study may have an immediate impact on the treatment of stroke.

4.5. Perturbing protein-protein interaction downstream of NR2B activation may be an alternative to NR2B blockage

Previous stroke clinical trials with NMDAR antagonists, including NR2B-selective blockers, have not seen success (Lee et al., 1999b;Kemp and McKernan, 2002;Ikonomidou and Turski, 2002b). In the present study, we also demonstrated that in the rat focal cerebral ischemia model, although NMDAR-mediated excitotoxic neuronal injuries following stroke are primarily mediated by NR2B-containing receptors, as NR2B-containing NMDAR-specific antagonist applied prior to the stroke onset significantly reduced ischemic brain damage, NR2B antagonist offers little protection when administered 4.5 h after the stroke onset. The reasons for this failure are still controversial. The rapid recovery of extracellular glutamate concentrations to pre-stroke levels (Benveniste et al., 1984;Ikonomidou and Turski, 2002a), which are not sufficient to activate extrasynaptic NMDARs, may partially account for this narrow therapeutic window. In most clinical settings, due to the time required to transport a patient to the hospital and obtain a definitive diagnosis, treatment is not usually possible until several hours after the stroke onset, which is most likely outside the window of efficacy for any NMDAR blockers including NR2B-selective antagonists. Thus, consistent with previous suspicions, our results suggest that the failure to administer NMDAR antagonists within

their efficacy window might be one of the major reasons for their failure in the clinical trials of stroke and neurotrauma conducted thus far.

Although the rapid recovery of glutamate levels following stroke makes it unfeasible for NR2B-specific antagonists to treat stroke, it takes time to transduce the pro-death signals following activation of NR2B-containing NMDARs. Therefore, an alternative that is able to disrupt the downstream signaling may provide an opportunity to prevent neuronal death. The NR2B9c peptide used in this study was derived from the last nine amino acids of the C-tail of NR2B subunit. Since both NR2A and NR2B C-tails contain the same binding motif (ESDV) in the very last four amino acids for the second PDZ domain on PSD-95, NR2B9c peptide is very likely to dissociate the binding of PSD-95 to NR2A as well. However, data from the Tymianski group suggest this peptide only selectively perturbs the interaction between NR2B and PSD-95. Comparison of the sequences of last nine residues of the NR2A C-tail and the NR2B9c peptide indicates there are two different residues between them. This incomplete homology of NR2B9c for NR2A C-tail may account for its ineffectiveness in perturbing the binding of PSD-95 to NR2A. Alternatively, NR2A may be more tightly bound to PSD-95 because it may preferentially associate with PSD-95 (van et al., 2004). Given that the NR2B9c peptide contains only nine amino acids and that it shares a high homology with NR2A C-tail but still does not interfere with the NR2A-PSD-95 interaction, this peptide may have few non-specific binding partners besides PSD-95. The high selectivity of NR2B9c is also indirectly confirmed by the lack of observable untoward effects in the experiments *in vivo*.

Consistent with the effects of the NR2B9c peptide on excitotoxicity *in vitro* (Aarts et al., 2002), our results suggested that it also has dramatic effects on ischemic brain damage *in*

vivo. This therapy is fundamentally different from blockade of NR2B-containing NMDARs in that it may occur without affecting NMDAR activity, and hence the NR2A-mediated cell survival signaling is intact. In addition, it is efficacious when carried out after the insult onset. All these suggest targeting of the NR2B-PSD-95 interaction is a practical strategy for treating stroke. Thus, this may represent a new direction in stroke therapeutics. Lastly, it is also probable that a similar approach could be used to modulate signals mediated by protein-protein interactions that lead to other human diseases.

4.6. Promising NMDAR-based neuroprotective therapies for ischemic brain damage

In this research project, we have shown that NMDARs are still on the center stage of excitotoxic injuries but also play a crucial role in neuronal survival. This functional differentiation is dictated by their subunit composition, i.e., NMDAR subtypes. According to these findings, here we proposed several NMDAR-based strategies to achieve neuroprotection from stroke-induced brain injury (Fig. 8).

If a stroke is predictable, blocking NR2B-containing NMDARs before stroke would constitute an ideal neuroprotective therapy. Through administration of a highly selective NR2B antagonist just before stroke attack, the overactivation of NMDARs containing NR2B during stroke can be inhibited, and hence the associated cell death signaling will not be initiated. In the meantime, the ensuing excessive release of glutamate will stimulate NR2A-containing NMDARs, which will in turn trigger the anti-apoptotic signaling pathway, and guard neurons against NMDAR-independent injuries during stroke. Regretfully, at present it is almost impossible to know when a stroke will occur; therefore, albeit appealing, this therapy may not be clinically useful. However, if a stroke

patient can receive medical attention within the therapeutic time window (usually less than 3 h), this therapy will still be beneficial. Unfortunately, in most clinical settings, stroke patients have missed out the optimal time window. This makes an effective post-ischemic therapy much more desirable. Theoretically, neuroprotection offered by selective activation of NR2A-containing NMDARs has no time limit and could counteract not only NMDAR-dependent but also NMDAR–independent neuronal death. Accordingly, stimulation NMDARs containing NR2A by highly specific NR2A agonists may be an optimal therapy for stroke patients at this stage. Alternatively, disruption of the signaling pathway downstream of NR2B activation with agents such as the NR2B C-tail interference peptide may also be efficacious. Since these two approaches are not occlusive, a comprehensive treatment that integrates NR2A activation and dissociation of NR2B-mediated cell death signaling could be more protective.

Fig. 8

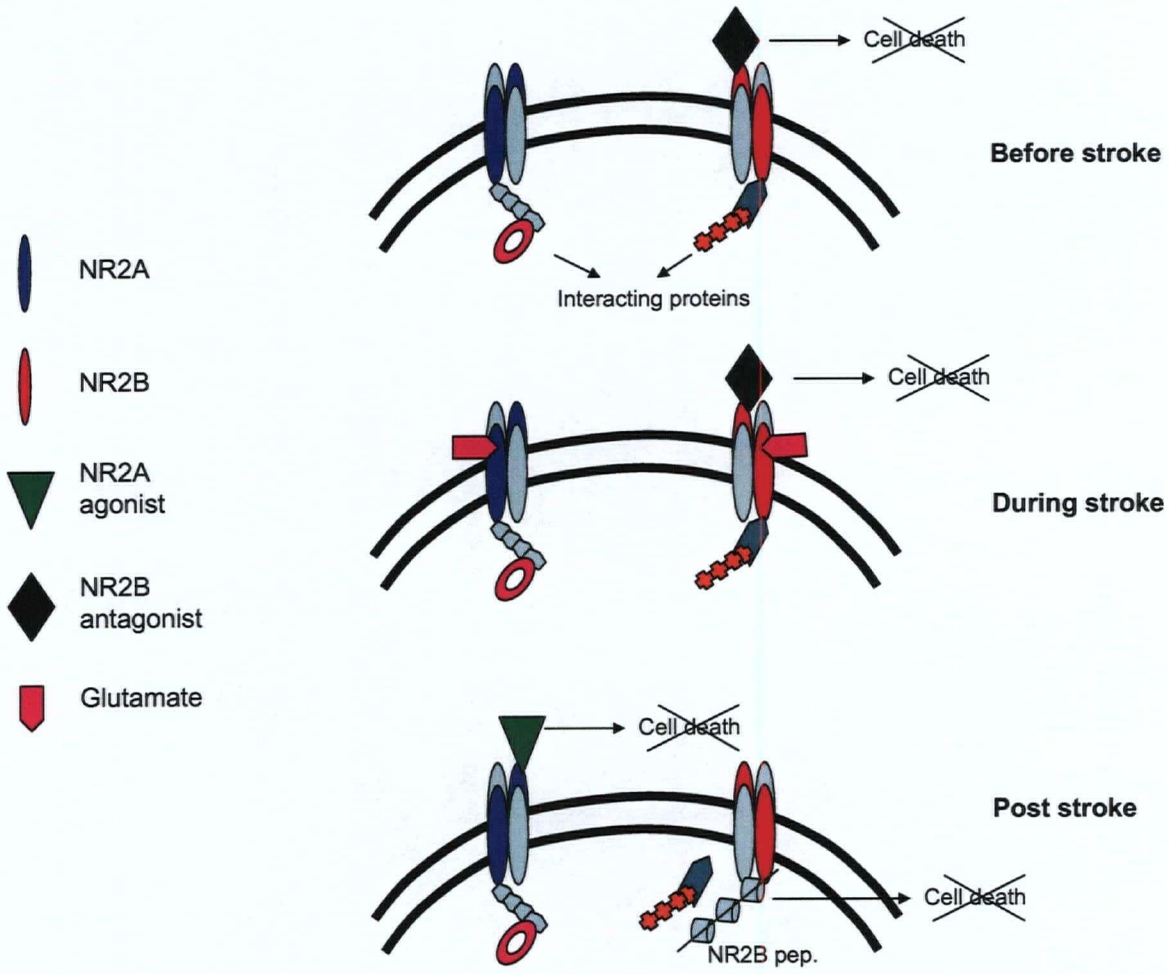

NR2A
NR2B
NR2A agonist
NR2B antagonist
Glutamate
Cell death
Interacting proteins
Before stroke
Cell death
During stroke
Cell death
NR2B pep.
Cell death
Post stroke

Fig. 8. **Schematic illustrates potential NMDAR-based therapies for stroke. Before stroke occurs, selective blockage of NR2B may be an ideal approach to prevent ischemic injury. NR2B-specific blockers can still be efficacious if administered timely during stroke. Following stroke onset, dissociation of the cell death signaling pathway downstream with interfering agents such as NR2B peptide (NR2B pep.) used in this study and/or post-stroke stimulation of NR2A with highly specific agonists may constitute feasible therapies for ischemic brain damage. These strategies may also apply to other neurodegenerative diseases in which excitotoxicity is involved.**

4.7. Future directions

We have shown here that NMDAR subtypes may play disparate roles in neuronal fate, and also demonstrated for the first time that selective activation of NR2A-containing NMDARs may exert neuroprotection. However, because of the limitation of time, there is still so much left to be studied. In the future, I wish to elucidate three major issues.

The first question I would like to address in the near future is the exact signaling pathways of NR2A- and NR2B-containing NMDARs. Although we have found that stimulation of these two NMDAR subtypes may activate opposing signaling pathways leading to cell survival or death, the mechanisms underlying this differentiation are still far from clear. However, only when these downstream pathways are fully understood can we develop suitable neuroprotective agents to promote cell survival and/or prevent cell death. There are several ways to achieve this goal, for example, imaging potential downstream mediators of these signaling pathways in live neurons following specific activation of a NMDAR subtype may provide insights into this issue.

Since we have shown in animals that selective activation of NR2A-containing NMDARs can greatly reduce brain injury, I would like to collaborate with industry and develop some highly specific NR2A agonists. The efficacy of such NR2A-selective agonists will then be examined in animals. If found to be able to reduce ischemic injury, they would move onto stroke clinical trials. In addition, as the NR2B C-tail peptide used in this study has shown amazing effects in attenuating brain injury, a clinical trial for this peptide to treat stroke should be under consideration. Hopefully, some new lines of drugs for treating stroke can be invented.

Finally, I want to determine the roles of other NMDAR subtypes such as NR2C- and

NR2D-containing NMDARs in excitotoxicity. In humans stroke mainly occurs in the forebrain; however, other regions in the central nervous system, such as brain stem, cerebellum and spinal cord, are also frequently attacked by stroke. The subunit composition of NMDARs in these regions is different from that in forebrain. Thus, determining the functions of the NMDAR subtypes other than NR2A- and NR2B-containing NMDARs is also critical in stroke therapeutics.

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APPENDIX

Part of the work presented in this thesis has been published in:

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with Tat38-48-dansyl (10 μM), devoid of the transduction domain, exhibited only background signal indicating no peptide uptake (Fig. 1B, right). Tat-NR2B9c-dansyl accumulation was detectable in neurons within 10 min of application, peaked during the next 20 min, and remained detectable for 5 hours after washing the peptide from the bath (Fig. 1C).

Next we determined whether Tat-NR2B9c could perturb preformed NMDAR–PSD-95 complexes by examining its effects on the coimmunoprecipitation of PSD-95 with NR2 subunits (*13*). The P2 membrane protein fraction of rat forebrain tissue, which is enriched in synaptic structures, was incubated with Tat-NR2B9c or with one of three controls: Tat38-48, the Tat transduction sequence conjugated to two alanine residues (Tat-AA), or a Tat-NR2B9c peptide in which the COOH-terminal tSXV motif contained a double point mutation (Lys-Leu-Ser-Ser-Ile-Glu-Ala-Asp-Ala; Tat-NR2B-AA) rendering it incapable of binding PSD-95 (*3*). None of these controls, each lacking an intact PDZ binding motif, reduced the coimmunoprecipitation of PSD-95 with NR2B. However, Tat-NR2B9c, in which the Ile-Glu-Ser-Asp-Val sequence is preceded by residues unique to the NR2B COOH-terminus, selectively reduced the coimmunoprecipitation of PSD-95 with NR2B (Fig. 1D), but not with NR2A (Fig. 1E). Thus, NR2A may be more tightly bound to PSD-95. Alternatively, the incomplete homology of Tat-NR2B9c for the NR2A COOH-terminus may make it less effective in perturbing PSD-95–NR2A binding (*13*).

Because synaptic and whole-cell NMDAR currents are unaffected when PSD-95 is lacking (*6, 7*), we examined NMDAR currents and Ca^{2+} fluxes when PSD-95 is dissociated. Bath application of Tat-NR2B9c (50 nM) to acute rat hippocampal slices had no effect on synaptic responses of CA1 neurons evoked by stimulation of the Schaffer collateral-commissural pathway (Fig. 2A). Tat-NR2B9c also had no effect on patch recordings in CA1 neurons of the primarily AMPAR (AMPA receptor)–mediated total excitatory postsynaptic current (EPSC) (Fig. 2B) (*15*), nor on the isolated NMDA component of the EPSC (Fig. 2C) (*13*). Moreover, pretreating cultured cortical neurons with Tat-NR2B9c or with pTat-PDZ1-2 (each at 50 nM) did not alter $^{45}Ca^{2+}$ uptake produced by applying NMDA (Fig. 2D). The rate of rise and peak levels of free intracellular calcium concentration ($[Ca^{2+}]_i$) in response to NMDA were also unaffected by Tat-NR2B9c (*13, 16*). CNQX (6-cyano-7-nitroquinoxaline-2,3-dione; 10 μM) and nimodipine (2 μM) were present in the extracellular solution in these and all further experiments in cultures, so as to isolate signaling to NMDARs and prevent secondary activation of AMPARs or of voltage-gated Ca^{2+} channels, respectively (*7, 17, 18*).

We next determined whether Tat-NR2B9c affected signaling events downstream of NMDAR activation. NMDA-evoked changes in guanosine 3′,5′-monophosphate (cGMP) level were measured as a surrogate measure of NO production by nNOS (*7, 19*). We focused on nNOS activity because it mediates neurotoxic sequelae of NMDAR activation (*5*) and, along with other signaling molecules bound to

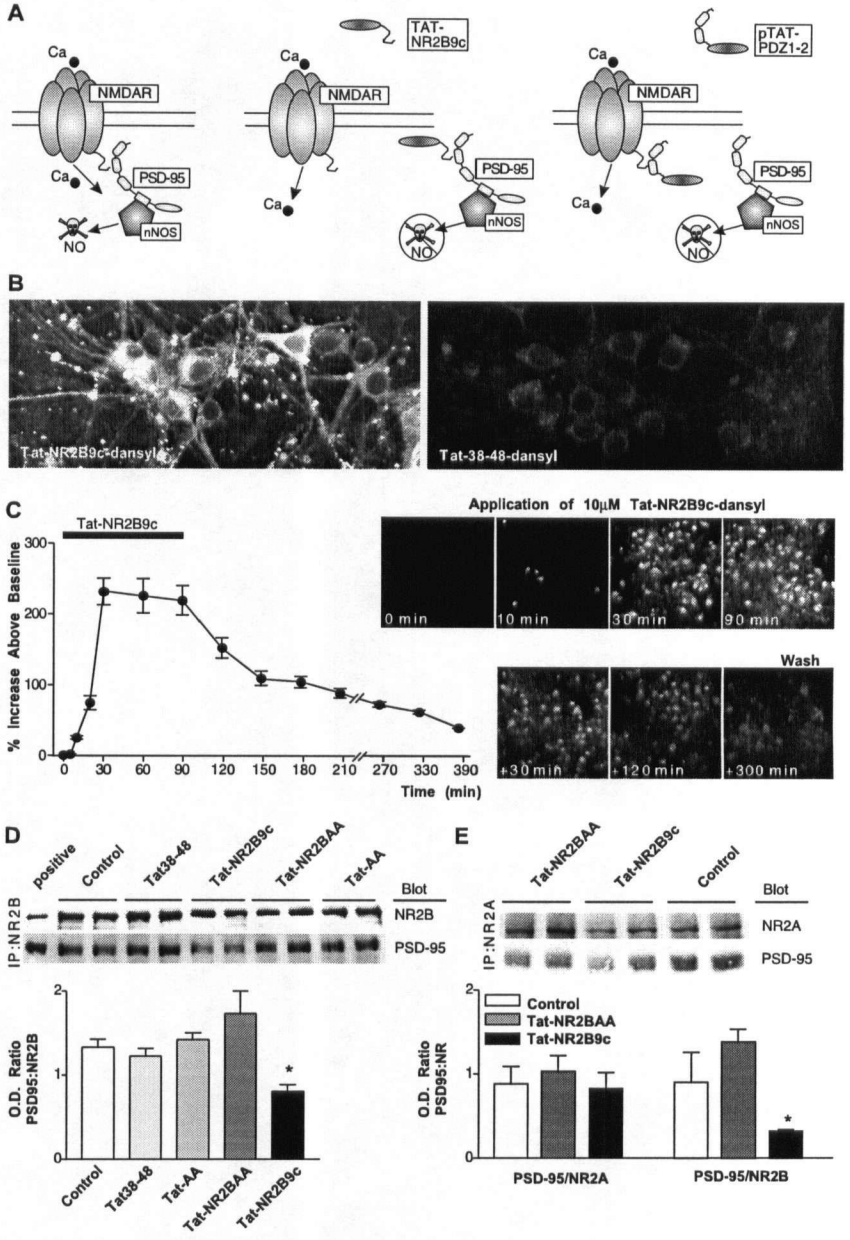

Fig. 1. Utility of Tat peptides in dissociating the NMDAR–PSD-95 interaction (**A**) The hypothesis: The NMDAR–PSD-95 complex (left panel) may be dissociated using Tat peptides fused either to the COOH-terminus of NR2B (Tat-NR2B9c; middle) or to the first and second PDZ domains of PSD-95 (pTat-PDZ1-2; right), thus reducing the efficiency of excitotoxic signaling. (**B**) Visualization of intraneuronal accumulation of Tat-NR2B9c-dansyl (10 μM) but not Tat38-48-dansyl (10 μM) 30 min after application to cortical cultures (excitation, 360 nm; emission, >510 nm; representative of five experiments). Fluorescence of cultures treated with Tat38-48-dansyl was similar to background. (**C**) Time course of Tat-NR2B9c-dansyl (10 μM) fluorescence after application to cortical cultures at room temperature (symbols: mean ± SE of four experiments). Inset: fluorescence images from a representative experiment. (**D** and **E**) Coimmunoprecipitation of PSD-95 with NR2 subunits in rat forebrain P2 membrane fractions treated with Tat peptides. (D) Tat-NR2B9c reduced the optical density (O.D.) ratio of PSD-95:NR2B by 37.6 ± 8.2% relative to controls. ANOVA, $F = 6.086$, $^*P = 0.0041$. (E) No significant effect on O.D. ratio of PSD-95:NR2A while reducing O.D. ratio of PSD-95:NR2B (ANOVA, $^*P < 0.01$) in same tissue extract. Top: Representative gels. Bottom: Means ± SE of four to eight experiments.

PSD-95, should be dissociated from NMDARs by Tat-NR2B9c. Cultured cortical neurons were pretreated with Tat-NR2B9c (50 nM), the noninteracting Tat-NR2B-AA (50 nM), or sham washes for 1 hour and then challenged with NMDA (0 to 1000 μM). NMDA produced a concentration-dependent increase in cGMP that was significantly suppressed (average of 39.5 ± 6.7%) by pretreating the cultures with Tat-NR2B9c, but not with Tat-NR2B-AA (Fig. 2E).

Although Tat-NR2B9c treatment did not affect NMDAR-mediated currents or Ca^{2+} fluxes, it interfered with NMDAR–PSD-95 binding and suppressed downstream NO signaling. Thus, we examined whether such treatment enhances neurons' resilience to NMDA toxicity. Cortical neuronal cultures were pretreated with sham washes, Tat-NR2B9c, or the noninteracting control Tat-NR2B-AA (each at 50 nM) for 1 hour, then exposed to NMDA (0 to 100 μM) for 1 hour followed by a 20-hour observation period (fig. S1, inset). Cell death at all NMDA concentrations was significantly reduced by Tat-NR2B9c pretreatment, whereas Tat-NR2B-AA was ineffective (fig. S1). Pretreatment with pTat-PDZ1-2 also attenuated NMDA neurotoxicity to a similar degree (fig. S1) (*13*), which suggested that targeting either side of the NMDAR–PSD-95 interaction (Fig. 1A) reduces excitotoxic damage.

Agents that block NMDAR activity are deleterious or ineffective in treating stroke in animals and humans (*9–11*). Because Tat-NR2B9c attenuates NMDA toxicity without blocking NMDARs, we reasoned that its application in the treatment of stroke would constitute an improvement over NMDAR blockers.

We first determined whether Tat-NR2B9c could be delivered into the brain in the intact animal. C57BL/6 mice (25 g) were injected intraperitoneally with a 500-μmol dose of either Tat-NR2B9c-dansyl or Tat38-48-dansyl as a cell-impermeant control. Coronal brain sections taken 1 hour after injection were examined by confocal microscopy for fluorescent peptide uptake (*13*). Brains from animals injected with Tat-NR2B9c, but not Tat38-48-dansyl, exhibited strong fluorescence in the cortex (fig. S2A) (*20*). Similar results were obtained with intravenous injection in rats (*21*), confirming that Tat-NR2B9c enters the brain upon peripheral administration.

Next, we examined whether pretreatment with Tat-NR2B9c would reduce stroke damage. Adult male Sprague-Dawley rats were subjected to transient middle cerebral artery occlusion (MCAO) for 90 min by the intraluminal suture method (*13, 22, 23*). Animals were pretreated by a single intravenous bolus injection with saline, the Tat-NR2B-AA control, or Tat-NR2B9c 45 min before MCAO (3 nmol/g). Body temperature, blood pressure, and blood gases were monitored and maintained throughout the experiment (table S1). The extent of cerebral infarction was measured 24 hours after MCAO onset (fig. S2C, inset). The postural reflex test (*24*) and the forelimb placing test (*25*) were used to grade neurological function on a scale of 0 to 12 (normal = 0; worst = 12) during MCAO (at 50 min) and 24 hours thereafter.

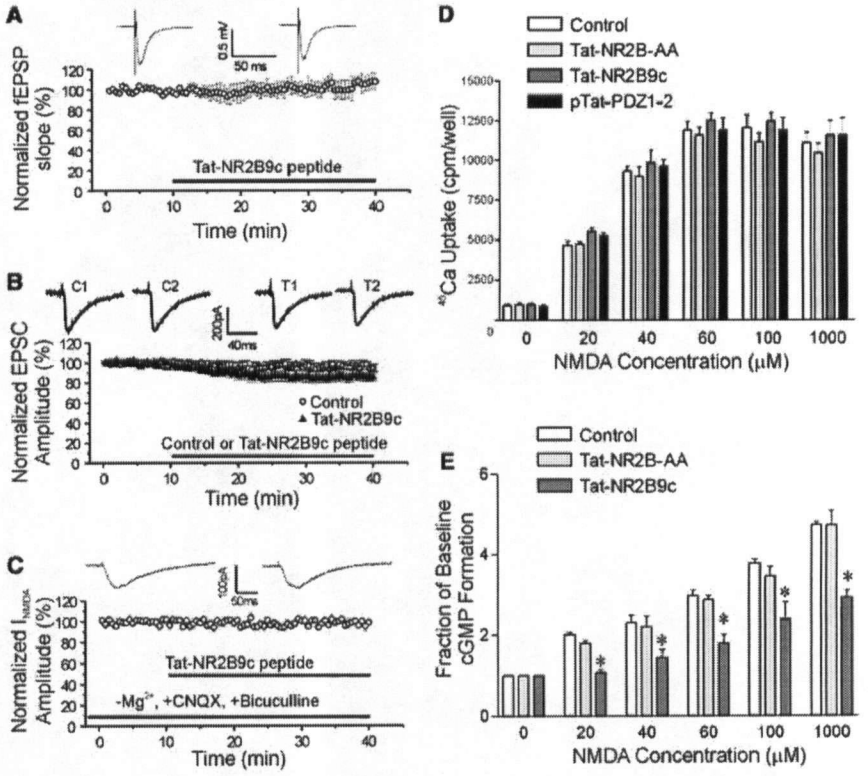

Fig. 2. Neurophysiological effects of Tat peptides (all at 50 nM). **(A)** Effect of Tat-NR2B9c on field EPSCs (fEPSCs) of CA1 neurons in acute hippocampal slices. **(B)** Effect of Tat-NR2B9c or Tat38-48 (control) on whole-cell EPSCs. **(C)** Effect of Tat-NR2B9c on the pharmacologically isolated NMDA component of the EPSC. **(D)** Effect of Tat-peptide pretreatment for 1 hour on NMDA-evoked $^{45}Ca^{2+}$ uptake in cortical cultures. **(E)** Effect of Tat-NR2B9c pretreatment for 1 hour on NMDA-evoked cGMP production in cortical cultures. Asterisk: differences from control and Tat-NR2B-AA at each NMDA concentration (Bonferroni *t* test, $P < 0.01$). Bars in (D) and (E) are means ± SE for 12 cultures in three separate experiments.

Pretreatment with Tat-NR2B9c produced a trend toward improvement in 24-hour neurological scores (fig. S2B). Moreover, the treatment reduced the total cerebral infarction volume by 54.6 ± 11.3% [fig. S2C(i); analysis of variance (ANOVA), $F = 7.289$, $P = 0.0048$]. This was largely accounted for by a 70.7 ± 11.2% reduction in cortical infarction [fig. S2C(ii), ANOVA, $F = 8.354$, $P = 0.0027$], thought to be largely caused by NMDAR-dependent mechanisms.

A stroke treatment with a single-bolus injection would be most therapeutically valuable if effective when given after the onset of ischemia. To evaluate whether Tat peptides could be neuroprotective when applied after insult in vitro, we first exposed cultured cortical neurons to an NMDA challenge (0 to 100 μM) for 1 hour, and then treated these cultures with the Tat peptides (all at 50 nM) described in the pretreatment study (fig. S1). Attenuation of NMDA toxicity in cultures treated with Tat-NR2B9c or pTat-PDZ1-2 was significant relative to cultures treated with control peptides (Fig. 3A).

Finally, we examined whether treatment with Tat-NR2B9c could attenuate ischemic neuronal damage in vivo when applied after stroke onset. Rats were subjected to transient MCAO for 90 min as before, and intravenous saline or Tat-peptide bolus (Tat-NR2B9c or Tat-NR2B-AA; 3 nmol/g) was injected 1 hour after MCAO onset (Fig. 3C, inset). Infarction volume and neurological outcome measurements were performed at times identical to the pretreatment study. Physiological parameters were monitored throughout the 24-hour experiment and were maintained equivalent between groups (table S2). Animals treated after MCAO with Tat-NR2B9c, but not with Tat-NR2B-AA or saline, exhibited a significant improvement in 24-hour neurological scores (Fig. 3B; ANOVA, $F = 17.25$, $P < 0.0001$). Treatment with Tat-NR2B9c reduced the volume of total cerebral infarction

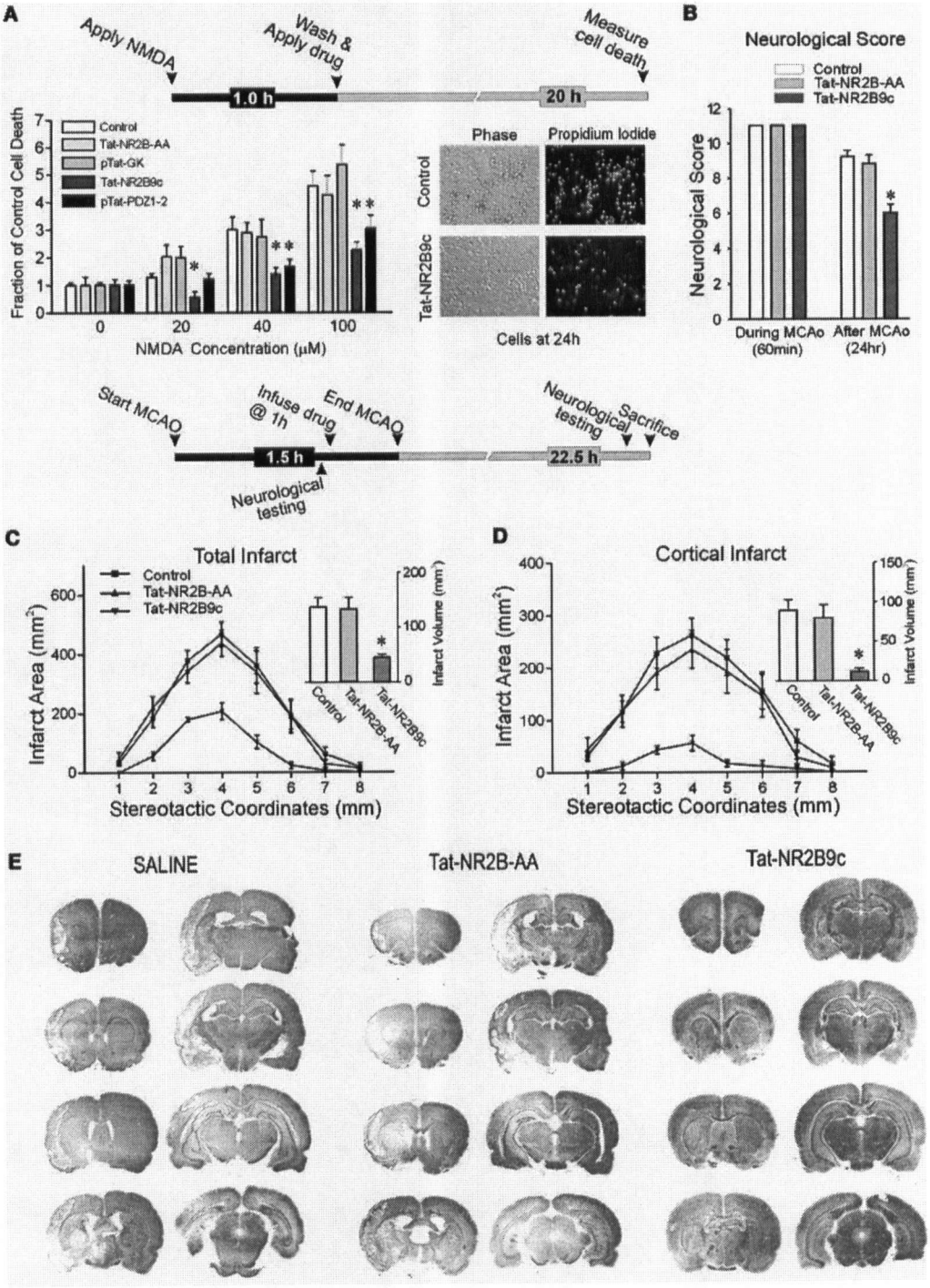

Fig. 3. Neuroprotection after insult by treatment with Tat-NR2B9c in vitro and in vivo. (**A**) Decreased excitotoxicity at 20 hours in cultured cortical neurons treated 1 hour after NMDA application with 50 nM Tat-NR2B9c or pTat-PDZ1-2. Bars indicate means ± SE for 12 cultures in three separate experiments. Asterisk: differences from control, Tat-NR2B-AA and pTat-GK (*13*) at each NMDA concentration (Bonferroni *t* test, $P < 0.005$). (Right) Representative phase contrast and propidium iodide fluorescence images of treated and control cultures 20 hours after challenge with 100 μM NMDA. (**B**) Composite neurological scores (see text) during and 24 hours after MCAO. Asterisk: difference from control and Tat-NR2B-AA (ANOVA; $F = 17.25$, $P < 0.0001$). (**C** and **D**) Treatment with Tat-NR2B9c (3 nmol/g, 9 animals) but not mutated Tat-NR2B-AA (8 animals) or saline controls (10 rats) significantly reduced (C) total infarct area and volume (inset) (ANOVA; $F = 12.0$, $P < 0.0005$) and (D) cortical infarct area and volume (inset) (ANOVA; $F = 12.64$, $P = 0.0001$), measured 24 hours after transient MCAO. Symbols and bars indicate means ± SE. (**E**) Representative appearance of hematoxylin and eosin–stained rat brain sections from each treatment group.

by 67.0 ± 3.7% (Fig. 3C; ANOVA, $F = 11.99$, $P = 0.0002$). Similar to the pretreatment study, this reduction was accounted for by an 87.0 ± 4.4% reduction in cortical infarction volume (Fig. 3, D and E; ANOVA, $F = 12.64$, $P < 0.0001$).

Our results show that introducing into cells an exogenous peptide containing the COOH-terminal nine amino acids of the NR2B NMDAR subunit has profound effects on signaling pathways downstream of NMDAR activation, on in vitro excitotoxicity, and on in vivo ischemic brain damage. The effects of this peptide are lost by mutating amino acids that are essential for mediating PDZ binding to PSD-95. Interfering with the interaction between NMDARs and PSD-95 may interrupt signaling downstream from NMDARs that leads to neuronal death. Because this occurs without affecting NMDAR activity, adverse consequences of blocking NMDARs are not expected. Efficacy after the insult onset suggests targeting of the NMDAR–PSD-95 interaction as a practical future strategy for treating stroke. It is probable that a similar approach could be used to modulate signals mediated by protein-protein interactions that lead to other human diseases.

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16. Ratios of peak $[Ca^{2+}]_i$ and rate of rise of $[Ca^{2+}]_i$ before and after Tat-NR2B9c application were 1.03 ± 0.03 and 0.99 ± 0.04, respectively (n = 11 neurons).
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Supporting Online Material

www.sciencemag.org/cgi/content/full/298/5594/846/DC1
Materials and Methods
Supplemental Online Text
Tables S1 and S2
Figs. S1 and S2

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Cancer Regression and Autoimmunity in Patients After Clonal Repopulation with Antitumor Lymphocytes

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We report here the adoptive transfer, to patients with metastatic melanoma, of highly selected tumor-reactive T cells directed against overexpressed self-derived differentiation antigens after a nonmyeloablative conditioning regimen. This approach resulted in the persistent clonal repopulation of T cells in those cancer patients, with the transferred cells proliferating in vivo, displaying functional activity, and trafficking to tumor sites. This led to regression of the patients' metastatic melanoma as well as to the onset of autoimmune melanocyte destruction. This approach presents new possibilities for the treatment of patients with cancer as well as patients with human immunodeficiency virus–related acquired immunodeficiency syndrome and other infectious diseases.

Immunotherapy of patients with cancer requires the in vivo generation of large numbers of highly reactive antitumor lymphocytes that are not restrained by normal tolerance mechanisms and are capable of sustaining immunity against solid tumors. Immunization of melanoma patients with cancer antigens can increase the number of circulating $CD8^+$ cytotoxic T lymphocyte precursor cells (pCTLs), but to date this has not correlated with clinical tumor regression, suggesting a defect in function or activation of the pCTLs (*1*).

Adoptive cell transfer therapies provide the opportunity to overcome tolerogenic mechanisms by enabling the selection and activation of highly reactive T cell subpopulations and by manipulation of the host environment into which the T cells are introduced. However, prior clinical trials, including the transfer of highly active antitumor T cell clones, failed to demonstrate engraftment and persistence of the transferred cells (*2–5*). Lymphodepletion can have a marked effect on the efficacy of T cell transfer therapy in murine models (*6–9*) and may depend on the destruction of regulatory cells, disruption of homeostatic T cell regulation, or abrogation of other normal tolerogenic mechanisms.

To determine whether prior lymphodepletion might improve the persistence and function of adoptively transferred cells, 13 HLA-$A2^+$ patients with metastatic melanoma received immunodepleting chemotherapy with cyclophosphamide and fludarabine for 7 days before the adoptive transfer of highly selected tumor-reactive T cells and high-dose interleukin-2 (IL-2) therapy (*10*) (Table 1). These patients all had progressive disease refractory to standard therapies, including high-dose IL-2, and eight patients also had progressive disease despite aggressive chemotherapy. The patients received an average of 7.8×10^{10} cells (range, 2.3×10^{10} to 13.7×10^{10}) and an average of nine doses of IL-2 (range, 5 to 12 doses). The T cells used for treatment were derived from tumor-infiltrating lymphocytes (TILs) and were rapidly expanded in vitro (*11*). All cultures were highly reactive when stimulated with an HLA-$A2^+$ melanoma or an autologous melanoma cell line (Table 1 and table S1).

Six of the 13 patients had objective clinical responses to treatment and four others demonstrated mixed responses, with significant shrinkage of one or more metastatic deposits (*11*). Objective tumor regression was seen in the lung, liver, lymph nodes, and intraperitoneal masses and at cutaneous and subcutaneous sites. Five patients, all with evidence of concomitant cancer regression, demonstrated signs of autoimmune melanocyte destruction, including four patients with vitiligo and one patient with anterior uveitis (Table 1). All patients recovered from treatment with absolute neutrophil counts greater than 500/mm^3 by day 11 after T cell infusion but with slower recovery of $CD4^+$ cells, as expected after fludarabine therapy (*12*).

To investigate the function and fate of the transferred T cell populations, T cell receptor (TCR) expression was examined using a pan-

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Supporting Online Material

Materials and Methods

Generation of fusion proteins. pTat-PDZ1-2 and pTat-GK fusion proteins were generated by insertion of PSD95 residues 65-248 encoding the PDZ 1 and 2, and residues 534-724 encoding the guanylate kinase-like domains, respectively, into pTAT-HA plasmids (generous gift of S. Dowdy, Washington University, St. Louis, MO). Fusion proteins contain a 6X His-tag, the protein transduction domain of HIV-1 Tat and a hemagglutinin-tag N-terminal to the insert. Plasmids were transformed into BL21(DE3)LysS bacteria (Invitrogen) and recombinant proteins were isolated under denaturing conditions on a Nickel-His column.

Preparation of rat brain proteins. Rat brain proteins were prepared under weakly denaturing conditions known to permit the NMDAR/PSD-95 interaction[1]. Adult (7-8W) wistar rat forebrains were removed and homogenized in ice-cold buffer (0.32M sucrose, 0.1mM Na3VO4, 0.1mM phenylmethyl sulfonyl fluoride, 0.02M p-nitrophenyl phosphate, 0.02M glycerol phosphate, and 5ug/ml each of antipain, aprotinin, and leupeptin). Homogenates were centrifuged at 800gr for 10min at 4°C. The supernatants were centrifuged at 11,000g at 4°C for 20min and the pellets (P2) were resuspended in homogenization buffer. P2 membranes, a fraction enriched with synaptic structures, were adjusted to 200ug protein/90ul with homogenization buffer, and DOC and Triton X-100 were added to final concentrations of 1% and 0.1% respectively. After 30 min at 37°C suspensions were centrifuged at 100,000 g_{av} for 10 min and the supernatants were used for co-immunoprecipitation.

Co-immunoprecipitation. Tat peptides (100 μM final concentration) were incubated with 200 μg of rat forebrain lysate for 1 hour at 37°C. NR2 subunits were precipitated from rat forebrain extracts using polyclonal rabbit antibodies. The anti-NR2A and anti-NR2B antibodies were generated against the C-terminal regions encompassing amino acid residues 934 -1203 of the NR2A protein and residues 935-1,455 of the NR2B protein, respectively. These antibodies selectively recognize their respective proteins. Proteins were then separated on 8% SDS-PAGE gels and probed with anti-NR2B or anti PSD-95 antibodies. Detection of proteins was achieved using HRP-conjugated secondary antibodies and enhanced chemiluminescence.

Electrophysiology. Recordings were made in 400 μm Hippocampal slices from 20-36 day old Sprague-Dawley rats perfused at room temperature with ACSF containing (in mM) 126 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.2 KH_2PO_4, 26 $NaHCO_3$ and 10 glucose and bubbled with 95%O_2/5%CO_2. Whole-cell recordings of CA1 neurons were performed using the "blind" method with an Axopatch-1D amplifier at holding potential –60 mV. Pipettes (4-5 MΩ) were filled with solution containing (mM): 135 CsCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 0.5 EGTA, 10 HEPES, 4 Mg-ATP, 0.2 GTP, and 5 QX-314, pH 7.4, 310 mOsm. Field potentials were recorded with glass micropipettes (2-4 MΩ) filled with ACSF placed in the stratum radiatum 60-80 μm from the cell body layer. Synaptic responses were evoked by stimulation (0.05 ms) of the Schaffer collateral-commissural pathway with a bipolar tungsten electrode in the presence of bicuculline methiodide (10 μM). For I_{NMDA} recording, Mg^{2+} was removed from and 20 μM CNQX was added in ACSF. Following 10-20 min base line recordings of EPSCs, I_{NMDA} and fEPSPs, Tat-peptides were applied in ACSF and recordings were continued for 30 min thereafter.

Calcium imaging. Ratiometric measurements of free intracellular calcium concentration $[Ca^{2+}]_i$ were performed in primary cultured neurons loaded with the fluorescent Ca^{2+}

indicator fura-2/AM as described[2]. NMDA (250 μM dissolved in Mg^{2+}-free solution) was applied by pressure ejection (1 sec, every 90 sec) from a micropipette placed near the cell soma. Evoked calcium responses were recorded before and after bath application of Tat-NR2B9c (50 nM, 20 min). Rate of rise of $[Ca^{2+}]_i$ was calculated from the time taken to rise from 5% to 95% of peak values. For each cell, peak ratio and rate of rise of $[Ca^{2+}]_i$ was averaged from 4 responses obtained before and 4 after Tat-NR2B9c application. Data were expressed as before:after ratios of responses obtained before and after peptide application.

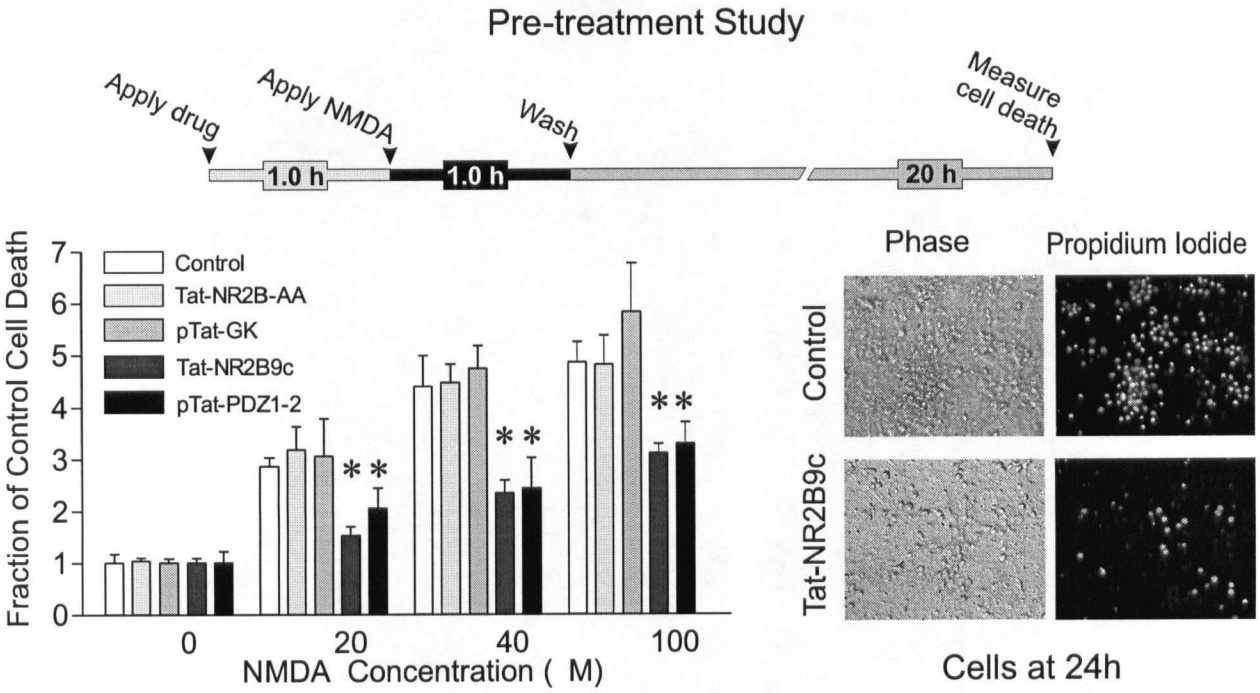
Brain perfusion and fluorescent Tat protein detection. The mice were perfused with fixative solution (3% paraformaldehyde, 0.25% glutaraldehyde, 10% sucrose, 10U/mL heparin in Saline) 1 hour after peptide injection. Brains were removed, frozen in 2-methylbutane at –42°C and 40μm sections were cut with a cryostat. Coronal sections were examined for dansyl fluorescence by UV-laser confocal microscopy.

Focal cerebral ischemia. Animals were fasted overnight and injected with atropine sulfate (0.5 mg/kg IP). After 10 minutes anesthesia was induced with 3.5% halothane in a mixture of nitrous oxide and oxygen (Vol. 2:1) and maintained with 0.8% halothane. Rats were orally intubated, mechanically ventilated, and paralyzed with pancuronium bromide (0.6 mg/kg IV). Body temperature was maintained at 36.5-37.5°C with a heating lamp. Polyethylene catheters in the femoral artery and vein were used to continuously record blood pressure and to sample blood for gas and pH measurements. Transient MCAO was achieved for 90 min by introducing a poly-L-lysine-coated 3-0 monofilament nylon suture into the circle of Willis via the internal carotid artery, effectively occluding the middle cerebral artery. This produces an extensive infarction encompassing the cerebral cortex and basal ganglia. All experimental manipulations and analyses of data were performed by individuals blinded to the treatment groups.

Supplemental Online Text.

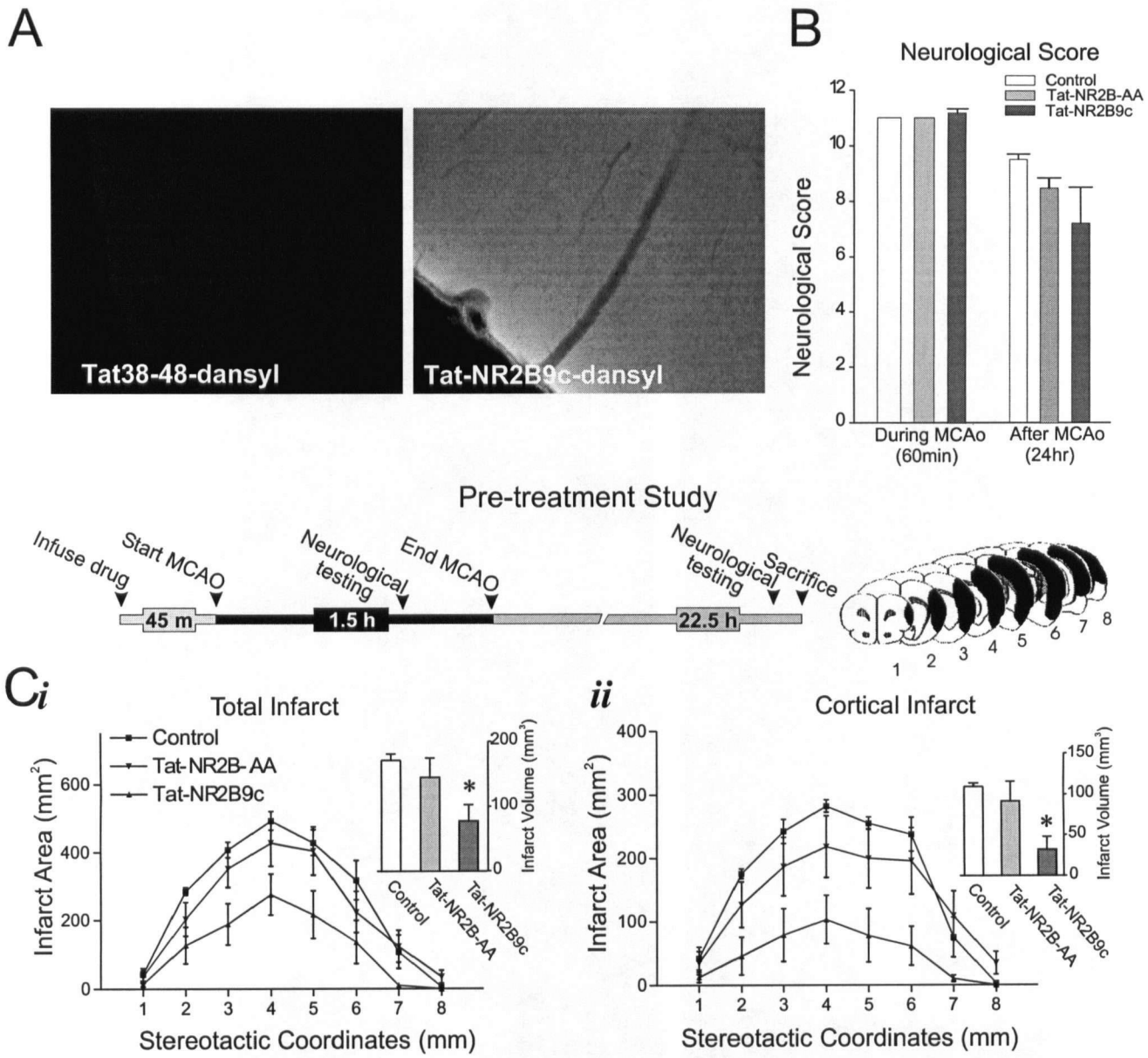
The Tat-NR2B9c peptide affected the interaction of PSD-95 with NR2B, but not NR2A subunits (Fig. 1E). Thus, amino acids upstream from the IESDV sequence common to the NR2A and NR2B c-terminus may indicate the binding efficiency of these subunits to PSD-95[3]. The factors governing these differential interactions remain to be elucidated. However, our findings indicate that perturbing PSD-95/NR2B binding is sufficient to treat excitotoxic and ischemic neuronal damage. This may indicate that NMDARs that mediate excitotoxicity preferentially contain NR2B over NR2A subunits, or that perturbing the interaction of PSD-95 with NR2B subunits is sufficient to suppress downstream excitotoxic signaling.

If Tat-NR2B9c suppresses NMDA excitotoxicity by interfering with the binding of NR2B to PSD-95 then interfering with this binding by an alternative means should also suppress the toxicity. We therefore also tested pTat-PDZ1-2, predicted to interfere with PSD-95 binding to NR2B and to permeate into the cells, though without effect on NMDA-evoked Ca^{2+} accumulation (Fig. 2D). Pre-treating the cultures with pTat-PDZ1-2 attenuated the neurotoxicity of NMDA to a similar degree as Tat-NR2B9c (Fig. S1). As a control we made and used pTat-GK, a Tat fusion protein containing residues 534-724 of PSD-95 comprising the carboxyl- terminal guanylate-kinase homology domain that lacks enzymatic activity[4]. pTat-GK, which is devoid of the necessary domains to bind NR2B, had no effect on the NMDA –evoked cell death (Fig. S1). Thus, interfering with the NMDAR/PSD-95 interaction using peptides that target either side of the interaction reduces *in vitro* excitotoxicity produced by NMDAR activation.



Supplemental Figure 1

Decreased excitotoxicity at 20h at all NMDA concentrations in cultured cortical neurons pre-treated with 50 nM Tat-NR2B9c or pTat-PDZ1-2 for 1h. Asterisk: differences from control, Tat-NR2B-AA and pTat-GK at each NMDA concentration (Bonferroni t-test, $p < 0.005$). Right panels: Representative phase contrast and propidium iodide fluorescence images of cultures 20h after challenge with 100 M NMDA with and without Tat-NR2B9c treatment. Bars indicate the mean ± S.E. for 12 cultures in 3 separate experiments.



Supplemental Figure 2

Neuroprotection by Tat-NR2B9c pretreatment in-vivo. (A) Detection of Tat-NR2B9c-dansyl but not Tat38-48-dansyl in the cortex of C57BL/6 mouse brain 1h after intraperitoneal injection (0.5 μmole total dose). Fluorescence of brains from animals treated with Tat-38-48-dansyl was similar to background. (B) Composite neurological scores (see text) during and 24h after MCAo. (C) Pre-treatment with 3 nmole/g Tat-NR2B9c but not mutated Tat-NR2B-AA or saline (control) significantly reduced (i) total infarct area and volume (inset), ANOVA; $F= 7.3$, $p<0.005$ and (ii) cortical infarct area and volume (inset), ANOVA; $F= 8.35$, $p<0.005$ measured 24h after transient MCAo. ($n = 6$ animals per group; symbols and bars indicate mean ± S.E). Infarct volume was calculated by analyzing the infarct area in 8 stereotactic coordinates of the brain as shown at right inset.

Supplemental Table 1: Physiological Variables in Pre-Treatment MCAO Study

Physiological Variables	**Control (n=6)**	**TAT-NR2BAA (n=6)**	**TAT-NR2B9c (n=6)**
Before anesthesia			
Body weight, g	269 ± 6	273 ± 7	271 ± 5
Before MCAo(45min)			
Body Temperature, ^{0}C	36.7 ± 0.07	36.7 ± 0.17	36.6 ± 0.21
MABP, mmHg	119 ± 4	115 ± 5	120 ± 9
Before MCAo(30min)			
Body Temperature, ^{0}C	36.8 ± 0.08	36.5 ± 0.12	36.7 ± 0.19
MABP, mmHg	107 ± 3	110 ± 4	76 ± 5*
Blood gases			
PH	7.44 ± 0.02	7.44 ± 0.02	7.44 ± 0.02
PO2, mmHg	104 ± 3	110 ± 7	123 ± 8
PCO2, mmHg	39.6 ± 1.3	39.1 ± 1.4	38.1 ± 1.4
Before MCAo(15min)			
Body Temperature, ^{0}C	36.9 ± 0.11	36.6 ± 0.15	36.7 ± 0.20
MABP, mmHg	111 ± 6	115 ± 5	90 ± 6*
During MCAo (5min)			
Body Temperature, ^{0}C	36.9 ± 0.03	36.6 ± 0.17	36.7 ± 0.16
MABP, mmHg	132 ± 6	135 ± 7	112 ± 9
Blood gases			
PH	7.44 ± 0.02	7.44 ± 0.02	7.44 ± 0.02
PO2, mmHg	118 ± 3	109 ± 4	112 ± 6
PCO2, mmHg	39.2 ± 0.6	39.6 ± 0.5	41.0 ± 1.3
During MCAo (15min)			
Body Temperature, ^{0}C	36.9 ± 0.09	36.7 ± 0.15	36.8 ± 0.23
MABP, mmHg	116 ± 9	111 ± 6	98 ± 6
After MCAo (15min)			
Body Temperature, ^{0}C	36.9 ± 0.09	36.8 ± 0.08	36.8 ± 0.12

After MCAo (24hr)			
Body Temperature, ^{0}C	36.6 ± 0.14	37.0 ± 0.25	36.5 ± 0.14
Body weight, g	238 ± 6	244 ± 6	250 ± 5

MABP: Mean arterial blood pressure

*** : P<0.05, Student's t-test**

Supplemental Table 2: Physiological Variables in Post- Treatment MCAO Study

Physiological Variables	Control (n=10)	TAT-NR2BAA (n=8)	TAT-NR2B9c (n=9)
Before anesthesia			
Body weight, g	314 ± 4	301 ± 5	306 ± 7
Before MCAo(15min)			
Body Temperature, ^{0}C	36.9 ± 0.07	36.7 ± 0.07	36.6 ± 0.07
MABP, mmHg	103 ± 4	103 ± 6	103 ± 5
Blood gases			
PH	7.43 ± 0.01	7.45 ± 0.01	7.43 ± 0.02
PO2, mmHg	113 ± 4	113 ± 4	105 ± 4
PCO2, mmHg	39.4 ± 1.0	37.9 ± 1.1	40.1 ± 1.0
During MCAo (15min)			
Body Temperature, ^{0}C	36.9 ± 0.07	36.7 ± 0.11	37.0 ± 0.07
MABP, mmHg	120 ± 5	121 ± 5	119 ± 8
Blood gases			
PH	7.44 ± 0.01	7.46 ± 0.01	7.43 ± 0.01
PO2, mmHg	113 ± 3	108 ± 2	111 ± 4
PCO2, mmHg	39.3 ± 0.7	48.0 ± 1.2	39.8 ± 0.9
During MCAo (60min)			
Body Temperature, ^{0}C	37.1 ± 0.21	37.0 ± 0.31	36.7 ± 0.11
MABP, mmHg	146 ± 5	149 ± 4	143 ± 5
During MCAo (65min)			
Body Temperature, ^{0}C	37.1 ± 0.16	37.0 ± 0.29	36.9 ± 0.08
MABP, mmHg	134 ± 6	136 ± 5	137 ± 4
After MCAo (15min)			
Body Temperature, ^{0}C	37.0 ± 0.09	36.9 ± 0.23	36.8 ± 0.08
MABP, mmHg	128 ± 6	116 ± 4	119 ± 4
After MCAo (24hr)			
Body Temperature, ^{0}C	36.6 ± 0.14	36.7 ± 0.27	36.4 ± 0.24
Body weight, g	276 ± 3	276 ± 6	279 ± 8

MCAo: Middle cerebral artery occlusion; MABP: Mean arterial blood pressure

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